



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07J 41/00, A61K 9/127, 31/575, C12N 15/63	A1	(11) International Publication Number: WO 97/45442 (43) International Publication Date: 4 December 1997 (04.12.97)
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(21) International Application Number: PCT/GB97/01426

(22) International Filing Date: 23 May 1997 (23.05.97)

(30) Priority Data:

9610944.2	24 May 1996 (24.05.96)	GB
9705498.5	17 March 1997 (17.03.97)	GB

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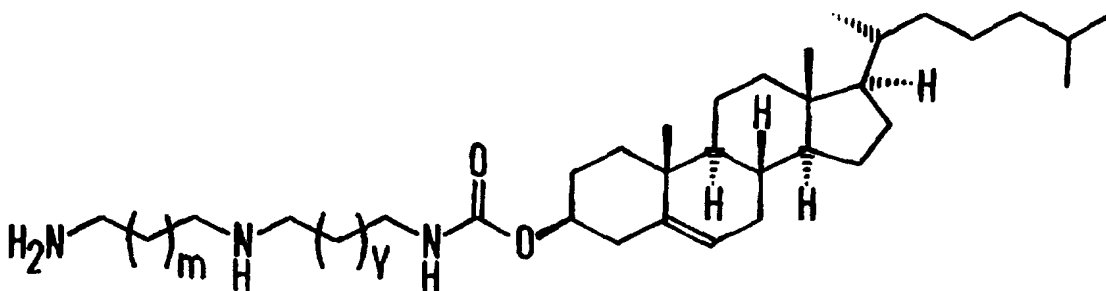
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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

(54) Title: POLYCATIONIC STEROL DERIVATIVES AS TRANSFECTION AGENTS



(57) Abstract

A compound capable of acting as a cationic lipid is described. The compound comprises a cholesterol group having linked thereto a head group; wherein the head group is more positive than the head group of DC-Chol.

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POLYCATIONIC STEROL DERIVATIVES AS TRANSFECTION AGENTS

The present invention relates to a compound. In addition, the present invention relates to processes for making the compound and to the use of that compound in therapy, in particular gene therapy (especially gene transfer).

One aspect of gene therapy involves the introduction of foreign nucleic acid (such as DNA) into cells, so that its expressed protein may carry out a desired therapeutic function.¹

Examples of this type of therapy include the insertion of TK, TSG or ILG genes to treat cancer; the insertion of the CFTR gene to treat cystic fibrosis; the insertion of NGF, TH or LDL genes to treat neurodegenerative and cardiovascular disorders; the insertion of the IL-1 antagonist gene to treat rheumatoid arthritis; the insertion of HIV antigens and the TK gene to treat AIDS and CMV infections; the insertion of antigens and cytokines to act as vaccines; and the insertion of β -globin to treat haemoglobinopathic conditions, such as thalassaemias.

Many current gene therapy studies utilise adenoviral gene vectors - such as Ad3 or Ad5 - or other gene vectors. However, serious problems have been associated with their use.² This has prompted the development of less hazardous, non-viral approaches to gene transfer.³

A non-viral transfer system of great potential involves the use of cationic liposomes.⁴ In this regard, cationic liposomes - which usually consist of a neutral phospholipid and a cationic lipid - have been used to transfer DNA⁴, mRNA⁵, antisense oligonucleotides⁶, proteins⁷, and drugs⁸ into cells. A number of cationic liposomes are commercially available^{4,9} and many new cationic lipids have recently been synthesised¹⁰. The efficacy of these liposomes has been illustrated by both *in vitro*⁴ and *in vivo*¹¹.

A neutral phospholipid useful in the preparation of a cationic liposome is *N*-[1-(2,3-

dioleoyloxy)propyl]-*N,N,N*-trimethyl ammonium chloride, otherwise known as "DOTMA". The structure of DOTMA is shown in Figure 1.

One of the most commonly used cationic liposome systems consists of a mixture of a neutral phospholipid dioleoylphosphatidylethanolamine (commonly known as "DOPE") and a cationic lipid, 3 β -[(*N,N*-dimethylaminoethyl)carbamoyl]cholesterol (commonly known as "DC-Chol")¹². The structure of DOPE is shown in Figure 2. The structure of DC-Chol is shown in Figure 3.

A lipid has been synthesised by reacting spermidine and cholesterol chloroformate in CH₂Cl₂ in the presence of *N,N*-diisopropylethylamine¹⁸. However, this resulted in a mixture of the lipid and the corresponding regio-isomeric lipid, which mixture proved inseparable by chromatography.

Despite the efficacy of the known cationic liposomes there is still a need to optimise the gene transfer efficiency of cationic liposomes in human gene therapy¹⁰.

According to one aspect of the present invention there is provided a compound capable of acting as a cationic lipid, the compound comprising a cholesterol group having linked thereto a head group; and wherein the head group is more positive than the head group of DC-Chol; but wherein the compound is not synthesised by reacting spermidine and cholesterol chloroformate in CH₂Cl₂ in the presence of *N,N*-diisopropylethylamine.

As indicated above, the head group of DC-Chol is Me₂N(CH₂)₂NH-.

According to another aspect of the present invention there is provided a process of preparing a compound according to the present invention comprising reacting a cholesterol group with a head group.

According to another aspect of the present invention there is provided a compound according to the present invention or a compound when prepared by the process of

the present invention for use in therapy.

According to another aspect of the present invention there is provided the use of a compound according to the present invention or a compound when prepared by the process of the present invention in the manufacture of a medicament for the treatment of a genetic disorder or a condition or a disease.

According to another aspect of the present invention there is provided a cationic liposome formed from the compound according to the present invention or a compound when prepared by the process of the present invention.

According to another aspect of the present invention there is provided a method of preparing a cationic liposome comprising forming the cationic liposome from the compound according to the present invention or a compound when prepared by the process of the present invention.

According to another aspect of the present invention there is provided a cationic liposome according to the present invention or a cationic liposome as prepared by the method of the present invention for use in therapy.

According to another aspect of the present invention there is provided the use of a cationic liposome according to the present invention or a cationic liposome as prepared by the method of the present invention in the manufacture of a medicament for the treatment of genetic disorder or condition or disease.

According to another aspect of the present invention there is provided a combination of a nucleotide sequence and any one or more of: a compound according to the present invention, a compound when prepared by the process of the present invention, a liposome of the present invention, or a liposome as prepared by the method of the present invention.

According to another aspect of the present invention there is provided a combination

according to the present invention for use in therapy.

According to another aspect of the present invention there is provided the use of a combination according to the present invention in the manufacture of a medicament
5 for the treatment of genetic disorder or condition or disease.

According to another aspect of the present invention there is provided a pharmaceutical composition comprising a compound according to the present invention or a compound when prepared by the process of the present invention
10 admixed with a pharmaceutical and, optionally, admixed with a pharmaceutically acceptable diluent, carrier or excipient.

According to another aspect of the present invention there is provided a pharmaceutical composition comprising a cationic liposome according to the present invention or a cationic liposome as prepared by the method of the present invention
15 admixed with a pharmaceutical and, optionally, admixed with a pharmaceutically acceptable diluent, carrier or excipient.

It is believed that a key advantage of the compound of the present invention is that
20 it can be used as a cationic lipid (amphiphile) in the preparation of a cationic liposome useful in gene therapy, in particular the transfer of nucleic acids (including genes and antisense DNA/RNA) into cells (*in vitro* and *in vivo*) to derive a therapeutic benefit.

The cholesterol group can be cholesterol or a derivative thereof. Examples of cholesterol derivatives include substituted derivatives wherein one or more of the cyclic CH₂ or CH groups and/or one or more of the straight-chain CH₂ or CH groups is/are appropriately substituted. Alternatively, or in addition, one or more of the cyclic groups and/or one or more of the straight-chain groups may be unsaturated.
25

30 In a preferred embodiment the cholesterol group is cholesterol. It is believed that cholesterol is advantageous as it stabilises the resultant liposomal bilayer.

Preferably the cholesterol group is linked to the head group *via* a carbamoyl linkage. It is believed that this linkage is advantageous as the resultant liposome has a low or minimal cytotoxicity.

5 Preferably the head group is a polyamine group. It is believed that the polyamine group is advantageous because it increases the DNA binding ability and efficiency of gene transfer of the resultant liposome.

10 In one embodiment, preferably the polyamine group is a naturally occurring polyamine. It is believed that the polyamine head-group is advantageous because the increased amino functionality increases the overall positive charge of the liposome. In addition, polyamines are known to both strongly bind and stabilise DNA¹⁴. In addition, polyamines occur naturally in cells and so it is believed that toxicological problems are minimised¹⁵.

15 Typical examples of suitable polyamines include spermidine, spermine, caldopentamine, norspermidine and norspermine. These polyamines are shown in Figure 4.

20 Preferably the polyamine is spermidine or spermine as these polyamines are known to interact with single or double stranded DNA. An alternative preferred polyamine is caldopentamine.

25 Thus, a preferred compound is spermidine linked to cholesterol *via* a carbamate linkage. This compound is shown in Figure 5. It is believed that the polyamino head group is advantageous for DNA condensation, the carbamate linkage is stable but biodegradable and the cholesteryl group imparts bilayer rigidity. The carbamate linkage may be part of, or an integral component of, the head group.

30 Another preferred compound is spermine linked to cholesterol *via* a carbamate linkage. Likewise, it is believed that the polyamino head group is advantageous for DNA condensation, the carbamate linkage is stable but biodegradable and the

cholesteryl group imparts bilayer rigidity.

Preferably the compound is in admixture with or associated with a nucleotide sequence.

5

The nucleotide sequence may be part or all of an expression system that may be useful in therapy, such as gene therapy.

Preferably the process comprises at least one step utilising aza-Wittig methodology.

10

Preferably the process comprises the use of trimethylphosphine.

Preferably the process comprises the use of a molecular sieve.

15

Preferably, the cationic liposome is formed from the compound of the present invention and a neutral phospholipid - such as DOTMA or DOPE. Preferably, the neutral phospholipid is DOPE.

20

In another embodiment, preferably two or more of the amine groups of the polyamine group of the present invention are separated by one or more groups which are not found in nature that separate amine groups of naturally occurring polyamine compounds (i.e. preferably the polyamine group of the present invention has unnatural spacing).

25

In summation, the present invention provides a compound capable of acting as a cationic lipid, the compound comprising a cholesterol group having linked thereto a head group; and wherein the head group is more positive than the head group of DC-Chol; but wherein the compound is not synthesised by reacting spermidine and cholesterol chloroformate in CH_2Cl_2 in the presence of *N,N*-diisopropylethylamine.

30

A preferred embodiment of the present invention is a compound capable of acting as a cationic lipid, the compound comprising a cholesterol group having linked thereto

5 a head group; wherein the head group is more positive than the head group of DC-Chol; wherein the cholesterol group is cholesterol; and wherein the head group is a polyamine group; but wherein the compound is not synthesised by reacting spermidine and cholesterol chloroformate in CH_2Cl_2 in the presence of *N,N*-diisopropylethylamine.

10 A more preferred embodiment of the present invention is a compound capable of acting as a cationic lipid, the compound comprising a cholesterol group having linked thereto a head group; wherein the head group is more positive than the head group of DC-Chol; wherein the cholesterol group is cholesterol; wherein the head group is a polyamine group; and wherein the cholesterol group is linked to the head group *via* a carbamoyl linkage; but wherein the compound is not synthesised by reacting spermidine and cholesterol chloroformate in CH_2Cl_2 in the presence of *N,N*-diisopropylethylamine.

15 A highly preferred embodiment of the present invention is a compound capable of acting as a cationic lipid, the compound comprising a cholesterol group having linked thereto a head group; wherein the head group is more positive than the head group of DC-Chol; wherein the cholesterol group is cholesterol; wherein the head group is a polyamine group; wherein the cholesterol group is linked to the head group *via* a carbamoyl linkage; and wherein the polyamine group is a naturally occurring polyamine, such as any one of spermidine, spermine or caldopentamine; but wherein the compound is not synthesised by reacting spermidine and cholesterol chloroformate in CH_2Cl_2 in the presence of *N,N*-diisopropylethylamine.

25 The present invention will now be described only by way of examples, in which reference is made to the following Figures:

30 Figure 1 which is a structure;
Figure 2 which is a structure;
Figure 3 which is a structure;
Figure 4 which is a series of structures;

- Figure 5 which is a structure;
Figure 6 which is a structure;
Figure 7 which includes a reaction scheme;
Figure 8 which includes a reaction scheme and a Table of results;
5 Figure 9 which includes a reaction scheme and a Table of results;
Figure 10 which includes a reaction scheme and a Table of results;
Figure 11 which includes a reaction scheme;
Figure 12 which includes a reaction scheme and a Table of results;
Figure 13 which includes a reaction scheme and a Table of results;
10 Figure 14 which includes a reaction scheme and a Table of results;
Figure 15 which includes a reaction scheme and a Table of results;
Figure 16 which includes a reaction scheme and a Table of results;
Figure 17 which includes a reaction scheme;
Figure 18 which includes a reaction scheme and a series of results;
15 Figure 19 which includes a reaction scheme and a series of results;
Figure 20 which includes a reaction scheme and a Table of results;
Figure 21 which includes a reaction scheme and a Table of results;
Figure 22 which presents a model;
Figure 23 which presents data from some studies;
20 Figure 24 which presents data from some studies;
Figure 25 which presents data from some studies;
Figure 26 which presents a reaction scheme;
Figure 27 which presents a reaction scheme;
Figure 28 which presents a reaction scheme; and
25 Figure 29 which presents some formulae.

GENERAL COMMENTS

- 30 In these studies DC-Chol was used as a template for the synthesis of more efficient gene transfer lipids, due to its good gene transfer ability¹² and low cytotoxicity¹³. In particular, the head group of DC-Chol was changed to a series of polyamine head-groups.

INITIAL STUDIES

Initially, a range of cationic lipids as shown in Figure 6 (where $m=1-3$, $n=1-2$; referenced as 2), with head groups based on the naturally occurring polyamine, spermidine ($m=3$, $n=3$; referenced as 3) were synthesised.

To prepare these compounds we used aza-Wittig methodology¹⁶, and so we made and used a range of suitably protected homologous azides and aldehydes (see Scheme 1 in Figure 7).

Our initial studies suggested that benzyloxycarbonyl protected aminoazides (see Figure 8, referenced as 4) would be suitable. These could be prepared in three steps and in excellent yield from aminoalcohols (see Figure 8, referenced as 3), by sequential *N*-benzyloxycarbonylation, mesylation and azidation.

This process and the yields therefrom are shown in Figure 8 (see Scheme 2 and Table 1).

The desired aldehyde could also be synthesised from aminoalcohols (see Figure 9, referenced as 5) in excellent yield, this time by *N*-protection with cholesteryl chloroformate to give alcohols (see Figure 9, referenced as 8), followed by Swern-type oxidation¹⁷ to aldehydes (see Figure 9, referenced as 9).

This process and the yields therefrom are shown in Figure 9 (see Scheme 3, Table 2).

The aldehydes (9) were isolated as white crystalline solids which, in contrast with analogous aldehydes with different protecting groups, proved to be extremely stable, easy to handle and could be stored for long periods of time without any discernible decomposition.

The aza-Wittig reaction between azides (4) and aldehydes (9) occurred smoothly in

THF to yield, after *in situ* reduction, protected polyamines (**10**) in excellent yield. We found that the use of trimethylphosphine rather than triphenylphosphine, led both to higher yields and lower reaction times. The use of molecular sieves also proved advantageous in obtaining consistently good yields, by eliminating adventitious water from the reaction system. Finally, removal of the benzyloxycarbonyl protecting group by hydrogenolysis yielded the desired lipids (**2**) in quantitative yield. These processes and the yields are shown in Figure 10 (see Scheme 4, Table 3).

Liposomes containing the new lipids could be prepared using the following general procedure which by way of example refers to lipid (**2**) and DOPE.

Lipid (**2**) (6 μ mol) and DOPE (298 μ l of a 10 mg/ml solution in CHCl_3 , 4 μ mol) were added *via* syringe to freshly distilled CH_2Cl_2 (5 ml) under a nitrogen atmosphere. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (5 ml of a 20 mM solution adjusted to pH 7.8) was added. The two phase mixture was sonicated for 3 minutes and the organic solvents were removed under reduced pressure. The resulting liposome suspension was then sonicated for a further 30 min, yielding liposomes with an average diameter of 150 nm (as determined using a Coulter N4 MD photon correlation spectrometer).

The new compounds yielded the correct analytical data by ^1H NMR, ^{13}C NMR, IR, MS and elemental analyses or HRMS.

This process therefore yields spermidine containing DC-Chol analogues from readily available starting materials, using aza-Wittig methodology. For these studies reference can also be made to Figures 11-21.

FURTHER STUDIES

The further studies involved the inclusion of higher homologue polyamines such as spermine and unusual polyamines such as caldopentamine into a lipid framework. It is believed that these lipids, when used within the confines of a liposome, provide a

DNA-condensing property that results in stronger, more stable liposome/DNA conjugates. Thus, we believe that these properties will improve the overall transfection of, for example, plasmid DNA into, for example, cell lines.

5 For these further studies reference can also be made to Figures 11-21.

Preparation of Methanesulfonate Esters

10 To a solution of dry alcohol (1.48 mmol) in dry CH_2Cl_2 (15 ml) was added triethylamine (0.62 ml, 4.4 mmol) and the solution stirred under nitrogen and cooled to around 0°C . At this point, methanesulfonyl chloride (0.29 ml, 3.7 mmol) in dry CH_2Cl_2 (5 ml) was added carefully and dropwise to the solution of alcohol. After the addition stirring was continued at 0°C for 10 minutes and then at RT for 10 minutes. Ice was then added carefully and the reaction quenched for 35 minutes. The CH_2Cl_2 15 was diluted five-fold and then extracted with sat. ammonium chloride (70 ml), water (50 ml) and brine (70 ml). The organic layer was then dried over anhydrous sodium sulfate and the solvent removed *in vacuo* to give a quantitative yield of the crude mesylate.

Preparation of Azides

20 To a stirred mixture of methanesulfonate ester (1.48 mmol), sodium azide (0.48 g, 7.4 mmol, 5 eq.) and sodium iodide (0.222 g, 1.48 mmol) under N_2 , was added by syringe dry DMF (10 ml) and the suspension heated to 80°C . This was maintained 25 for two hours, and then allowed to cool to room temperature. The slurry was then filtered over CeliteTM and the DMF removed under high vacuum to give a pale yellow oil.

30 The pale yellow oil was then redissolved in diethylether (100 ml) and washed with water (3 x 60 ml) and brine (80 ml). The organic layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give the colourless azide. The azide was then purified further by flash column chromatography (ether/acetone 4-30%) to give the

pure azide.

Preparation of Bromides

5 To a mixture of methanesulfonate ester (1 mmol) and sodium bromide (0.515 g, 5 mmol, 5 eq.) was added dry DMF (10 ml) *via* syringe and the solution heated at 80°C for two hours, and then allowed to cool to room temperature and the slurry filtered over Celite™, and the DMF removed *in vacuo*. The bromide was then redissolved in diethylether (50 ml) and washed with water (3 x 30 ml) and brine (50
10 ml). The organic extract was dried over anhydrous Na₂SO₄, and the solvent removed *in vacuo* to give a colourless oil. The bromide was then purified by flash column chromatography (diethylether 20%/petrol 80% → diethylether) to give pure bromide.

Mono-N-Alkylation with Bromides

15 To a stirred solution of bromide (1 mmol) and 4-amino-1-butanol (0.36 g, 4 mmol, 4 eq.) in anhydrous DMF (10 ml) under N₂, was added anhydrous K₂CO₃ (0.276 g, 2 mmol, 2 eq.) and sodium iodide (15 mg, 0.1 mmol, 0.1 eq.). The reaction was stirred for 72 hours at room temperature, until reaction complete. The DMF was
20 evaporated *in vacuo* and dried exhaustively under high vacuum. The resulting oily solid was redissolved in CH₂Cl₂ (100 ml) and washed with water (4 x 50 ml).

The combined aqueous extracts were then washed with CH₂Cl₂ (40 ml) and the CH₂Cl₂ extracts dried over anhydrous Na₂SO₄. The CH₂Cl₂ was evaporated *in vacuo*
25 to give a pale yellow oil.

Aza-Wittig Reaction

30 To a suspension of flame-dried 4Å molecular sieves (500 mg) and toluene-dried azide (1.08 mmol) in anhydrous THF (9 ml) under a nitrogen atmosphere, was added dropwise a solution of trimethylphosphine (1.2 ml of a 1.0 M solution in THF, 1.19 mmol, 1.1 eq.) *via* syringe.

After stirring at room temperature for one hour, the aldehyde (1.19 mmol, 1.1 eq.) in THF (2 ml) was added dropwise *via* cannula and the reaction mixture stirred for 18 hours. At this point, the THF was evaporated under nitrogen, and the resulting slurry resuspended in dry ethanol (7 ml). To this solution was added sodium borohydride (4.32 ml of 0.5 M solution in diglyme, 2.16 mmol, 2 eq.) *via* syringe and the reaction stirred for a further 24 hours at room temperature.

After this, the suspension was filtered over a short pad of Celite™, and the diglyme removed *in vacuo*. Exhaustive drying prior to work up was carried out, and the resulting oil redissolved in CH₂Cl₂ (120 ml) and treated with sat. sodium bicarbonate (100 ml), water (120 ml) and brine (120 ml). The organic fraction was dried over anhydrous Na₂SO₄ and the solvent removed *in vacuo* to give a pale yellow oil and the crude product purified by flash column chromatography.

15 Hydrogenolysis

To a solution of Z-protected lipid (0.52 mmol, 1 eq.) in ethanol (6 ml) was added cyclohexene (2.11 ml, 20.8 mmol, 40 eq.) followed by palladium on activated carbon (10%, 277 mg, 0.5 eq.) under nitrogen blanket. The solution was then refluxed under nitrogen and the reaction followed by TLC (CH₂Cl₂/MeOH/NH₃ 92:7:1).

After two hours, the catalyst was carefully filtered off under nitrogen, washed with fresh ethanol and the solvent evaporated *in vacuo* to give a white solid.

25 Preparation of Silanols

To a cooled, stirred solution of alcohol (0.93 mmol), triethylamine (0.39 ml, 2.8 mmol) and DMAP (11.4 mg, 0.093 mmol, 0.1 eq.) in dry CH₂Cl₂ (1.5 ml) was added dropwise a solution of *tert*-butylchlorodiphenylsilane (0.6 ml, 2.33 mmol, 2.5 eq.) in dry CH₂Cl₂ (1.5 ml). The solution was stirred at room temperature for three to five hours at which point CH₂Cl₂ (45 ml) was added to the reaction mixture and then extracted with sat. sodium bicarbonate (70 ml) and brine (70 ml).

The CH₂Cl₂ extracts were dried over anhydrous Na₂SO₄ and the solvent removed *in vacuo* to give a pale yellow oil. This was purified by flash column chromatography.

N-Protection of Aminosilanol

To an ice-cooled aminosilanol (0.85 mmol) and triethylamine (0.24 ml, 1.7 mmol, 2 eq.) in CH₂Cl₂ (4 ml) was added dropwise a solution of phenylmethoxycarbonyl chloride (0.26 ml, 1.7 mmol, 2 eq.) in CH₂Cl₂ (1 ml). The resulting solution was stirred at room temperature for 18 hours. The solution was then diluted with CH₂Cl₂ (30 ml), washed with saturated ammonium chloride (40 ml) and brine (40 ml). The CH₂Cl₂ extract was then dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo* to give a pale yellow oil. The compound was purified by flash column chromatography.

Preparation of Aldehydes

To a stirred solution of ethanedioyl dichloride (0.80 ml, 9.3 mmol, 1.5 eq.) in CH₂Cl₂ (20 ml) at -78°C under a nitrogen atmosphere was added a solution of DMSO (1.35 ml, 18.6 mmol, 3 eq.) in CH₂Cl₂ (20 ml) *via* cannula over a time period of 15 minutes. The resulting solution was stirred for a further 20 minutes after which time a solution of alcohol (6.2 mmol, 1 eq.) in CH₂Cl₂ (60 ml) was added dropwise *via* cannula. After a further 30 minutes *N,N,N*-diisopropylethylamine (3.25ml, 18.6 mmol, 3 eq.) was added dropwise and the solution warmed slowly to room temperature.

The pale yellow solution was then washed with saturated ammonium chloride (150 ml), saturated sodium bicarbonate (80 ml) and water (100 ml). The aqueous extracts were combined and washed with CH₂Cl₂ (100 ml). The CH₂Cl₂ extracts were combined, washed with brine (100 ml) and dried over anhydrous sodium sulfate. The solvent was then evaporated *in vacuo* to give a pale yellow solid. The crude aldehyde was then purified by flash column chromatography (ether) to a white solid.

Deprotection of Silanols

To a solution of silanol (1 mmol) in THF (5 mmol) was added carefully tetrabutyl ammonium fluoride (TBAF) (1.1 mmol) and the solution stirred for two to three hours. The solution was then diluted with CH₂Cl₂ (50 ml) and sat. sodium bicarbonate (50 ml) added. The organic phase was collected, washed with water (50 ml) and brine (60 ml). The organic layer was then dried over anhydrous sodium sulfate, and the solvent removed *in vacuo*. The pale yellow oil was then purified by flash column chromatography.

ADDITIONAL STUDIES

For these particular studies reference is made *inter alia* to Figures 22-28.

In particular, Figure 22 is the putative alignment of DC-Chol 1 and DOPE 2 in cationic liposome bilayer.

Figure 23 is an example of *in vitro* optimisation of pCF1- β Gal plasmid transfection of CFT1 cells *in vitro* using complexes of pCF1- β Gal and cationic liposomes formulated from DC-Chol analogue 8f' and DOPE 2 (1:1) molar ratio. CFT1 cells were transfected with an array of different cationic liposome and plasmid ratios in a 96-well plate. The extent of transfection in each well was determined after 2 days by measuring the levels of β -galactosidase expression. Plasmid DNA concentration is expressed as the concentration of nucleotides assuming an average nucleotide FWt of 330. Cationic liposome concentration is expressed in terms of the concentration of the constituent DC-Chol analogue 8f' alone.

Figure 24 presents rank order of DC-Chol polyamine analogues transfecting CFT1 cells *in vitro* with the pCF1- β Gal plasmid. Gene delivery activity is expressed as a proportion of the activity measured with standard liposomes containing DC-Chol 1 and DOPE 2. The data shown are the averages of four separate experiments, each performed in triplicate.

The ratios in curved brackets refer to the DC-Chol analogue:DOPE 2 molar ratio used to formulate the liposomes. The numbers in square brackets refer to compound serial numbers. Where appropriate DC-Chol analogue name abbreviations are also included (see experimental).

5

Figure 25 presents the rank order of DC-Chol polyamine analogues transfecting the lungs of female BALB/c mice with the pCF1-CAT plasmid. Mice were instilled with a solution of the plasmid (4 mM nucleotide concentration) and the optimal quantity and ratio of cationic liposome, in a total volume of 100 μ l. Gene delivery activity was determined as a function of chloramphenicol acetyl transferase activity in lung homogenates after 2 days.

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Data points were from separate experiments with each optimal formulation tested in 4 BALB/c mice. The ratios in curved brackets refer to the DC-Chol analogue:DOPE 2 molar ratio. The numbers in square brackets refer to compound serial numbers. Where appropriate DC-Chol analogue name abbreviations are also included (see experimental).

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Extra studies have shown that the gene delivery efficiency of DC-Chol/DOPE liposomes is equivalent to the performance of liposomes containing DC-Chol analogue 8c'.^[32]

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In order to improve to DC-Chol/DOPE liposomes, a simple model for the association of DC-Chol 1 and DOPE 2 in the bilayer of a cationic liposome was devised (Figure 22). This was based upon the proposed behaviour of cholesterol in bilayer membranes^[26] and a liposome model proposed by Felgner and co-workers.^[27]

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Carbon atoms C-1 to C-9 of the oleoyl side chains of DOPE 2 pack against the four fused cholesterol rings of DC-Chol 1 so that the phosphate ester group of DOPE and the protonated tertiary amine functionality of DC-Chol 2 are aligned and neutralise each other. The positive charge of the liposome then derives from the protonated ethanolamine side chain of DOPE 2. On the basis of the recent transfection

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experiments in mouse and human^[24, 25] it was thought that increased transfection efficiency could best be achieved by constructing either DC-Chol or DOPE polyamine analogues which would be expected to interact with DNA more strongly. Since DOPE is sensitive to oxidation of the oleoyl cis- double bonds, we considered that it would be more appropriate to synthesise polyamine analogues of DC-Chol instead. The model (Figure 22) suggested that the methylene group spacing between carbamoyl and the first amine functional group of a given DC-Chol polyamine analogue should be two or at most three, in order to maintain charge complementation with DOPE 2. The preferred DC-Chol polyamine analogues (i.e. polyamine analogues of 3DF-[*N*-(*N*',*N*'-dimethylaminoethane) carbamoyl]cholesterol) were designed with this constraint in mind.

The syntheses of triamine analogues of DC-Chol were carried out as follows.

Initially, two different *N*-cholesteryloxycarbonylaminoaldehydes 3' were prepared by *N*-protection of aminoalcohols 4' with cholesteryl chloroformate followed by Swern-type oxidation^[28] of protected alcohols 5' to give the aldehydes 3' (see Scheme A in Figure 26). Typically speaking aminoaldehydes can be quite unstable and prone to polymerisation but the steric stabilisation of the *N*-cholesteryl moiety resulted in crystalline compounds which could be stored for extended periods of time without any discernible decomposition.

N-Benzyloxycarbonyl protected aminoazides 6' were then prepared in three steps from aminoalcohols 4' using sequential *N*-benzyloxycarbonylation, mesylation and azidation (Scheme A). Finally, azides 6' were coupled to aminoaldehydes 3' using aza-Wittig methodology^[29, 30] giving protected DC-Chol triamine analogues 7' which were stored at this stage. In our hands, aza-wittig coupling reactions were found to be more efficient with trimethylphosphine rather than the customary triphenylphosphine, in line with literature precedent.^[29] Also, the elimination of adventitious water with activated molecular sieves proved helpful to obtain consistently high yields.^[29] Just prior to any gene delivery studies, protecting groups were removed by catalytic transfer hydrogenolysis, to give triamine analogues 8' (Scheme A) in 44-77% overall yield.

The syntheses of tetramine analogues of DC-Chol were carried out in the following way. Several different *N*-protected diaminoalcohols 9' were prepared by mono *N*-alkylation of aminoalcohols 4' with *N*-benzyloxycarbonyl protected aminoalkylbromides 10'. Bromides 10' were themselves prepared in three steps from aminoalcohols by sequential *N*-benzyloxycarbonylation, mesylation and bromination (see Scheme B in Figure 27). Customarily, mono-*N*-alkylations of primary amines are considered to be difficult to control. Nevertheless, a combination of steric crowding in the reactants and mild reaction conditions are now being found increasingly to prevent over-*N*-alkylation occurring.^[31] Temporary protection of the alcohol functional groups of 9' as *t*-butyldiphenylsilylethers, followed by *N*-benzyloxycarbonylation and fluoride promoted desilylation gave bonafide di-*N*-benzyloxycarbonyl protected diaminoalcohols which were then converted into diaminoazides 11' by mesylation followed by azidation (Scheme B). Finally, protected DC-Chol tetramine analogues 12' were formed by coupling azides 11' to cholesteryl-aminoaldehydes 3' by means of the the aza-Wittig procedure once more (Scheme B). As for the preparation of 8' (Scheme A), protecting groups were removed just prior to transfection studies by catalytic transfer hydrogenolysis giving tetramine analogues 13' (Scheme B) in 12-38% overall yield. Gratifyingly, we found that the mono-*N*-alkylation procedure could be used equally well to prepare *N*-cholesteryloxycarbonyl-diaminoaldehyde 14' from *N*-cholesterylalcohol 5' via bromide 15' (see Scheme C in Figure 28). As a result, several fully protected DC-Chol pentamine analogues 16' could be prepared by aza-Wittig coupling of diaminoaldehyde 14' to di-*N*-benzyloxycarbonyl protected diaminoazides 11'. DC-Chol pentamine analogues 17' were then prepared by hydrogenolysis of the protecting groups in the usual way (Scheme C).

The ability of cationic liposomes containing the different DC-Chol polyamine analogues to mediate gene delivery was analysed both *in vitro* and *in vivo*. Cationic liposomes were formulated by hydrating a dried lipid film, containing DC-Cholanalogue and DOPE 2 in an appropriate molar ratio of either 1:0, 1:1, 1:2 or 2:1 respectively, and vortex mixing.^[32] Cationic liposome/plasmid DNA complexes were then prepared by adding appropriately diluted cationic liposome

suspensions into equal volumes of aqueous plasmid DNA solutions at 30°C and allowing the mixture to equilibrate to ambient temperature over 15 min.^[32] *In vitro* studies were then performed with immortalised cystic fibrosis airway epithelial (CFT1) cells followed by *in vivo* studies in which cationic liposome/plasmid DNA complexes were instilled intranasally into the lungs of female BALB/c mice.^[32] Typically, cationic liposome gene delivery was first optimised *in vitro* so as to establish the best molar ratio of cationic liposome to plasmid DNA (DNA concentration was expressed as nucleotide concentration) as well as the best absolute quantities of both, as illustrated (Figure 23). This optimised combination was then tested *in vivo*. The *in vitro* results are shown (Figure 24). Six DC-Chol analogue containing liposomes proved to confer significant improvements on gene delivery efficiency over and above DC-Chol/DOPE liposomes formulated in a similar way. The analogues were 8a', 8b', 8e', 8f', 13a' and 17b', the first four being triamines, the fifth a tetramine and the sixth a pentamine. With one exception, 8f', these polyamine analogues contain inter-nitrogen methylene group spacings not normally associated with the natural polyamines spermidine 18' (see for example Figure 29), spermine 19' (see for example Figure 29) and pentamine 20' (see for example Figure 29) upon which these structures are based. *In vivo* (Figure 25), the best DC-Chol analogues were 13c', 13f' and especially 17a'. Both 17a' and 13c' also contain unnatural methylene group spacings.

Of the best *in vivo* analogues, 17a' works about 100-fold more effectively in mouse lung than DC-Chol 1 and approx 500-fold relative to plasmid DNA alone (Figure 25). Only one other cytofectin has been reported to function at this level of efficacy *in vivo*, namely the lipid 67 21' (see Figure 29), a "T" shaped tetramine analogue of DC-Chol.^[32] None of the other reported cytofectins appear to be close to this level of *in vivo* efficacy with the possible exception of (B1)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis-(dodecyloxy)-1-propanaminium bromide (GAP-DLRIE) 22' (see Figure 29) which has been reported to work about 100 fold better than plasmid DNA alone.^[33] Other cytofectins have been reported, but frequently have either not yet been used *in vivo* or else with rather poor results.^[21, 34, 35]

Analogues 8f' and 13f' have been reported previously, either without synthetic characterisation details^[32] or else as impure mixtures known as SpdC and SpC respectively.^[34] In the former case, *in vitro* and *in vivo* gene delivery behaviours were found to be comparable with our results reported here.^[32] In the latter case, SpC was reported not to work well and to be relatively toxic.^[34] Our data with analogue 13f' do not support these observations.

Experimental Procedure

The synthetic procedures used to make the DC-Chol polyamine analogues are documented below.

Scheme A

Reagents and conditions: i, CH₂Cl₂ (0.2M), CholOC(O)Cl (0.45 eqv), 5 h, 98-99%; ii, (a) CH₂Cl₂ (0.1M) (COCl)₂ (1.5 eqv), DMSO (3 eqv), -78°C, 15 min; (b) 5', 15 min; (c) i-Pr₂NEt (3 eqv) to r.t., 93-97%; iii, (a) CH₂Cl₂ (0.2M), PhCH₂OC(O)Cl (0.45 eqv), 10 h; (b) CH₂Cl₂ (0.2M), Et₃N (3 eqv), CH₃SO₂Cl (2.5 eqv), 0°C to r.t., 15 min; (c) DMF (0.15 M), NaN₃ (5 eqv), NaI, 80°C, 2 h, 68-87%; iv, (a) 6', THF (0.5M), 4 Å molecular sieves, PMe₃ (1.15 eqv), 30 min; (b) 3' (1.1 eqv), 3h; (c) EtOH (0.5 M), NaBH₄ (2eqv), 20 h, 72-90%; v, EtOH (0.2M), c-C₆H₁₀ (20 eqv), 10% Pd(C) (0.5 eqv), reflux, 30 min, 99%.

Scheme B

Reagents and conditions: i, (a) CH₂Cl₂ (0.2M), PhCH₂OC(O)Cl (0.45 eqv), 10 h; (b) CH₂Cl₂ (0.2M), Et₃N (3 eqv), CH₃SO₂Cl (2.5 eqv), 0°C to r.t., 15 min; (c) DMF (0.15 M), NaBr (5 eqv), 80°C, 2 h, 68-87%; ii, 10', DMF (0.1M), 4' (5 eqv), K₂CO₃ (2 eqv), NaI (0.3 eqv), 24-72 h, 74-88%; iii, (a) CH₂Cl₂ (0.2M), Et₃N (1.5 eqv), TBDPSCl (1.2 eqv), DMAP (0.1 eqv), 09AC to r.t., 2-4 h; (b) CH₂Cl₂ (0.2M), PhCH₂OC(O)Cl (1.5 eqv), Et₃N (1.5 eqv), 6-10 h; (c) THF (0.5M), TBAF (1.1 eqv), 4- 5 h; (d) CH₂Cl₂ (0.2M), Et₃N (3 eqv), CH₃SO₂Cl (2.5 eqv), 0°C to r.t., 15 min;

(e) DMF (0.15M), NaN₃ (5 eqv), NaI, 80°C, 2 h, 37-70%; iv, (a) 11', THF (0.5M), 4 Å molecular sieves, PMe₃ (1.15 eqv), 30 min; (b) 3' (1.1 eqv), 3h; (c) EtOH (0.5 M), NaBH₄ (2 eqv), 20 h, 64-71%; v, EtOH (0.2M), c-C₆H₁₀ (20 eqv), 10% Pd(C) (0.5 eqv), reflux, 30 min, 99%.

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Scheme C

Reagents and conditions: i, (a) CH₂Cl₂ (0.2M), Et₃N (3 eqv), CH₃SO₂Cl (2.5 eqv), 0°C to r.t., 15 min; (b) DMF (0.15 M), NaBr (5 eqv), 80°C, 2h, 68-87%; ii, (a) 15', DMF (0.1M), 4' (5 eqv), K₂CO₃ (2 eqv), NaI (0.3 eqv), 24-72 h; (b) CH₂Cl₂ (0.2M), Et₃N (1.5 eqv), TBDPSCl (1.2 eqv), DMAP (0.1 eqv), 0°C to r.t., 2-4 h; (c) CH₂Cl₂ (0.2M), PhCH₂OC(O)Cl (1.5 eqv), Et₃N (1.5 eqv), 6-10 h; (d) THF (0.5M), TBAF (1.1 eqv), 4-5 h; (e) CH₂Cl₂ (0.1M) (COCl)₂ (1.5 eqv), DMSO (3 eqv), -78°C, 15 min; (f) di *N*-protected diaminoalcohol, 15 min; (g) i-Pr₂NEt (3 eqv) to r.t., 53-66%; iii (a) 11', THF (0.5M), 4 Å molecular sieves, PMe₃ (1.15 eqv), 30 min; (b) 14' (1.1 eqv), 3h; (c) EtOH (0.5 M), NaBH₄ (2 eqv), 20 h, 56-74%; iv, EtOH (0.2M), c-C₆H₁₀ (20 eqv), 10% Pd(C) (0.5 eqv), reflux, 30 min, 99%.

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Data

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Representative analytical data presented for the most effective analogues *in vitro* (triamine 8e', tetramine 13a' and pentamine 17b') and *in vivo* (tetramine 13f' and pentamine 17a')

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4-aza-*N*¹-cholesteryloxycarbonyl-1,7-heptanediamine (ACH) [B178] 8e'

ν_{\max} (CH₂Cl₂) 3347, 2937, 2905, 2868, 1698, 1534, 1467, 1379 and 1264 cm⁻¹; δ_H (300 MHz) 5.76 (1H, br s, NHCO), 5.22 (1H, m, H-6'), 4.33 (1H, m, H-3'), 3.08 (2H, m, H-1), 2.65 (2H, t, J 6.5 Hz, H-7), 2.53 (2H, t, J 6.5 Hz, H-3), 2.41 (2H, m, H-5), 2.24-2.12 (2H, m, H-4'), 1.90-1.69 (5H, m, H-2', H-7', H-8'), 1.55-0.93 (28H, m, H-2, H-4, H-6, H-1', H-9', H-11', H-12', H-14' to H-17', H-20', H-22' to H-25', NH₂), 0.88 (3H, s, H-19'), 0.79 (3H, d, J 6.5 Hz, H-21'), 0.73 (6H, dd,

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J 6.0 and 0.5 Hz, H-26' and H-27'), 0.55 (3H, s, H-18'); δ_c (75 MHz) 156.29 (NHC(O)O), 139.77 (C-5'), 122.24 (C-6'), 73.85 (C-3'), 56.59 (C-14'), 56.09 (C-17'), 49.92 (C-9'), 47.61 (C-1), 47.52 (C-3), 42.20 (C-4'), 40.22 (C-5), 39.66 (C-16'), 39.42 (C-24'), 38.57 (C-7), 36.94 (C-2), 36.44 (C-22'), 36.11 (C-8'), 35.71 (C-20'), 33.04 (C-7'), 31.78 (C-6), 28.14 (C-2'), 27.87 (C-25'), 24.19 (C-12'), 23.78 (C-15'), 22.74 (C-23'), 22.49 (C-26'), 20.96 (C-11'), 19.25 (C-19'), 18.65 (C-21'), 11.77 (C-18'); m/z (FAB) 544 (M+H)⁺, 369 (Chol)⁺, 95, 43 (Found: (M+H)⁺, 544.4885. C₃₄H₆₂N₃O₂ requires (M+H)⁺, 544.4842).

10 **N¹-cholesteryloxy-carbonyl-3,7-diaza-1,9-nonanediamine (CDAN) [B198] 13a'**

ν_{\max} (CH₂Cl₂) 3584-3245, 2937, 2868, 1695, 1538, 1469, 1379, 1251, 1133 and 1014 cm⁻¹; δ_H (400 MHz) 5.82 (1H, br s, NHCO), 5.23 (1H, m, H-6'), 4.33 (1H, m, H-3'), 3.54-2.55 (16H, m, H-1 to H-4, H-6 to H-9, H₂N), 2.21-2.09 (2H, m, H-4'), 1.97-1.73 (5H, m, H-2', H-7', H-8'), 1.55-0.99 (23H, m, H-5, H-1', H-9', H-11', H-12', H-14' to H-17', H-20', H-22' to H-25'), 0.88 (3H, s, H-19'), 0.78 (3H, d, J 6.0 Hz, H-21'), 0.74 (6H, d, J 6.5 Hz, H-26' and H-27'), 0.55 (3H, s, H-18'); δ_c (100 MHz) 156.38 (NHC(O)O), 139.70 (C-5'), 122.30 (C-6'), 73.99 (C-3'), 56.56 (C-14'), 56.05 (C-17'), 51.91 (C-1), 49.88 (C-9'), 47.95 (C-2), 42.18 (C-4'), 41.12 (C-8), 39.63 (C-16'), 39.40 (C-24'), 38.53 (C-2), 36.90 (C-1'), 36.42 (C-22'), 36.08 (C-8'), 35.69 (C-20'), 31.75 (C-7'), 29.62 (C-5), 28.12 (C-2'), 27.86 (C-25'), 24.17 (C-12'), 23.75 (C-15'), 22.73 (C-23'), 22.48 (C-26'), 20.94 (C-11'), 19.24 (C-19'), 18.62 (C-21'), 11.76 (C-18'); m/z (FAB) 573 (M+H)⁺, 544, 513, 460, 369 (Chol)⁺, 215, 95 (Found: (M+H)⁺, 573.5139. C₃₅H₆₅N₄O₂ requires (M+H)⁺, 573.5108).

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N¹⁰-cholesteryloxy-carbonyl-4,8,13-triaza-1,16-hexadecanediamine(CTAH)[B222] 17b'

ν_{\max} (CH₂Cl₂) 3344, 2936, 2855, 1700, 1536, 1468, 1379, 1265, 1122 and 1028 cm⁻¹; δ_H (300 MHz) 5.69 (1H, br s, NHCO), 5.22 (1H, d, J 4.0 Hz, H-6'), 4.33 (1H, m, H-3'), 3.20 (2H, m, H-1), 2.63 (2H, t, J 6.5 Hz, H-3), 2.55-2.35 (12H, m, H-5, H-8, H-10, H-12, H-14, H-16), 2.23-2.08 (7H, m, H-4, H-9, H-13, H-4', NH₂),

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1.90-1.63 (5H, m, H-2', H-7', H-8'), 1.57-0.90 (31H, m, H-2, H-6, H-7, H-11, H-15, H-1', H-9', H-11', H-12', H-14' to H-17', H-20', H-22' to H-25'), 0.87 (3H, s, H-19'), 0.78 (3H, d, J 6.5 Hz, H-21'), 0.73 (6H, d, J 6.5 Hz, H-26' and H-27'), 0.55 (3H, s, H-18'); δ_c (75 MHz) 156.27 (NHC(O)O), 139.79 (C-5'), 122.24 (C-6'), 73.86 (C-3'), 56.58 (C-14'), 56.06 (C-17'), 49.92 (C-9'), 49.62 (C-1), 49.72 (C-3), 49.62 (C-12), 47.52 (C-14), 42.20 (C-4'), 39.65 (C-16'), 39.42 (C-24'), 38.56 (C-8), 36.93 (C-1'), 36.45 (C-22'), 36.09 (C-8'), 35.69 (C-20'), 31.78 (C-7'), 28.14 (C-2'), 27.88 (C-25'), 27.74 (C-2), 24.19 (C-12'), 23.74 (C-15'), 22.75 (C-23'), 22.49 (C-26'), 20.95 (C-11'), 19.26 (C-19'), 18.64 (C-21'), 11.77 (C-18'); m/z (FAB) 672 (M+H)⁺, 570, 539, 369 (Chol)⁺, 84 (Found: (M+H)⁺, 672.6205. C₄₁H₇₈N₅O₂ requires (M+H)⁺, 672.6156).

N¹-cholesteryloxy-carbonyl-4,9-diaza-1,12-dodecanediamine (CDAD) [B185] 13f

ν_{\max} (CH₂Cl₂) 3349, 2937, 2868, 1697, 1468, 1378 and 1253 cm⁻¹; δ_H (400 MHz) 5.70 (1H, br s, NHCO), 5.27 (1H, m, H-6'), 4.38 (1H, m, H-3'), 3.2 8-3.14 (6H, m, H-1, H-4, H-9, NH₂), 2.71 (2H, t, J 6.5 Hz, H-12), 2.63-2.52 (8H, m, H-3, H-5, H-8, H-10), 2.33-2.17 (2H, m, H-4'), 1.93-1.85 (5H, m, H-2', H-7', H-8'), 1.77-1.07 (29H, m, H-2, H-6, H-7, H-11, H-1', H-9', H-11', H-12', H-14' to H-17', H-20', H-22' to H-25'), 1.03 (3H, s, H-19'), 0.97 (3H, d, J 6.0 Hz, H-21'), 0.77 (6H, dd, J 5.0 and 1.5 Hz, H-26' and H-27'), 0.59 (3H, s, H-18'); δ_c (100 MHz) 156.25 (NHC(O)O), 139.77 (C-5'), 122.26 (C-6'), 73.87 (C-3'), 56.57 (C-14'), 56.04 (C-17'), 49.90 (C-1), 49.60 (C-9'), 49.47 (C-3), 47.40 (C-10), 42.19 (C-4'), 39.63 (C-16'), 39.40 (C-24'), 38.55 (C-8), 36.92 (C-5), 36.44 (C-22'), 36.08 (C-8'), 35.69 (C-20'), 31.77 (C-7'), 28.13 (C-2'), 27.87 (C-25'), 27.53 (C-2), 24.18 (C-16), 23.74 (C-12'), 22.73 (C-23'), 22.48 (C-26'), 20.94 (C-11'), 19.25 (C-19'), 18.62 (C-21'), 11.76 (C-18'); m/z (FAB) 615 (M+H)⁺, 539, 369 (Chol)⁺, 57 (Found: (M+H)⁺, 615.5626. C₃₈H₇₁N₄O₂ requires (M+H)⁺, 615.5577).

***N*¹⁵-cholesteryloxycarbonyl-3,7,12-triaza-1,15-pentadecanediamine(CTAP)[B232]
17a'**

ν_{\max} (CH₂Cl₂) 3568-3295, 2937, 1690, 1537, 1467, 1380, 1130 and 1019 cm⁻¹; δ_H
 (300 MHz) 5.76 (1H, br s, NHCO), 5.22 (1H, m, H-6'), 4.32 (1H, m, H-3'), 3.21
 (2H, m, H-15), 2.65 (2H, t, J 5.5 Hz, H-13), 2.56-2.45 (12H, m, H-1, H-2, H-4,
 H-6, H-8, H-11), 2.18-2.05 (2H, m, H-4'), 1.97-1.67 (10H, m, H-3, H-7, H-12,
 H-2', H-7', H-8', NH₂), 1.59-0.91 (29H, m, H-5, H-9, H-10, H-14, H-1', H-9',
 H-11', H-12', H-14' to H-17', H-20', H-22' to H-25'), 0.86 (3H, s, H-19'), 0.77
 (3H, d, J 6.5 Hz, H-21'), 0.72 (6H, dd, J 6.0 and 1.0 Hz, H-26' and H-27'), 0.53
 (3H, s, H-18'); δ_C (75 MHz) 156.24 (NHC(O)O), 139.77 (C-5'), 122.21 (C-6'), 73.81
 (C-3'), 56.57 (C-14'), 56.05 (C-17'), 49.91 (C-9'), 49.67 (C-15), 48.20 (C-13),
 42.19 (C-4'), 39.64 (C-16'), 39.40 (C-24'), 38.56 (C-2), 36.92 (C-1'), 36.43
 (C-22'), 36.08 (C-8'), 35.68 (C-20'), 31.77 (C-7'), 28.12 (C-2'), 27.86 (C-25'),
 27.69 (C-14), 27.63 (C-5), 24.17 (C-12'), 23.74 (C-15'), 22.73 (C-23'), 22.48
 (C-26'), 20.94 (C-11'), 19.25 (C-19'), 18.63 (C-21'), 11.76 (C-18'); *m/z* (FAB) 658
 (M+H)⁺, 539, 369 (Chol)⁺, 95, 84 (Found: (M+H)⁺, 658.6056. C₄₀H₇₆N₅O₂
 requires (M+H)⁺, 658.5999).

IN VITRO AND IN VIVO TESTS

For the *in vitro* and *in vivo* tests, a dried lipid film containing the given DC-Chol
 analogue and DOPE 2 (in either a 1:0, 1:1, 1:2 or 2:1 respective molar ratio), was
 hydrated for 10 min in sterile pyrogen-free water and then liposomes were produced
 by 2 min vortex mixing. The average diameter was between 200-400 nm.^[32]
 Cationic liposomes containing DC-Chol 1 and DOPE 2 were formulated in the same
 way described previously.^[24, 36]

Cationic liposome/plasmid DNA complexes were then prepared as follows.

Both cationic liposome suspensions and DNA (either pCF1- β Gal plasmid expressing
 β -galactosidase or pCF1-CAT expressing chloramphenicol acetyl transferase)^[32]

solutions were separately pre-incubated for 5 min at 30°C, before being diluted to the appropriate final concentrations and then combined. Usually, cationic liposome suspensions were added to an approx. equal volume of plasmid DNA solutions. Complexes were allowed to equilibrate for a minimum of 15 min at ambient temperature and used within 2 h of preparation. *In vitro* and *in vivo* gene delivery assays were then performed as described previously using CFT1 cells and female BALB/c mice respectively.^[32]

DISCUSSION

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In theory, genetic trait analysis will eventually be able to identify all the genetic loci which cause or contribute towards disease. With this information, corrective gene(s) may be identified which if introduced into the appropriate organs and cells of the body *in vivo* should correct the basic pathophysiological defect of the disease. This is the basic concept of gene therapy. Such a simple approach should be capable of curing the disease in contrast to most conventional pharmaceutical approaches which typically treat symptoms only.

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However, introducing potentially corrective gene(s) is not straightforward. Whilst naked DNA may be administered under certain circumstances, for the most part a delivery vehicle or vector is required to effect efficient gene delivery. Several physical, chemical and virus-based vector systems are known but none are sufficiently efficacious for general use in human gene therapy. In spite of this, some vectors are showing some promise, in particular cationic liposome-based gene transfer systems.^[21]

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Cationic liposomes are heterogeneous, lipid vesicles typically formed from either a single cationic amphiphile (sometimes known as a cytofectin) or more commonly from a combination of a cationic amphiphile and a neutral lipid. They mediate gene delivery by interacting electrostatically with negatively charged DNA sequences forming complexes which may enter cells by endocytosis^[22] or phagocytosis^[23] and then release DNA for expression in the cell nucleus.^[21] We have shown that cationic liposomes, formed from the cationic amphiphile 3 β -[N-(N',N'-dimethyl-aminoethane)-

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carbamoyl]cholesterol (DC-Chol) 1 and the neutral phospholipid dioleoyl L- α -phosphatidylethanolamine (DOPE) 2, were able to transfect the lungs of mice *in vivo*.^[24] Since then, some preparatory human clinical trials have been performed using similar DC-Chol/DOPE cationic liposomes.^[25]

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Both sets of experiments represent a proof of principle demonstrating that gene therapy with cationic liposomes is possible. However, both sets of experiments also showed that DC-Chol/DOPE cationic liposomes are unlikely to be efficient enough at gene delivery for general use in human gene therapy. Moreover, it is difficult to make improvements in the absence of any understanding of cationic liposome structure/activity relationships.

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The present invention seeks to improve the earlier studies. In addition, the present invention seeks to understand some of the underlying chemical principles behind liposome-mediated gene delivery.

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In this regard, a systematic series of DC-Chol analogues were made which could be incorporated into cationic liposomes and evaluated for gene delivery.

In conclusion, we have developed a flexible synthetic route to DC-Chol polyamine analogues which has allowed us to identify analogues with optimised methylene group spacing between amine functional groups for both *in vitro* and *in vivo* gene delivery. On the whole, so far we have found that unnatural spacing appears to work better. Without wishing to be bound by theory, perhaps, such polyamines have a slightly weakened interaction with DNA which facilitates the release of DNA into the cytoplasm, after transfer of the cationic liposome/DNA complex across the outer cell membrane.

20

25

At present, evidence so far suggests that our preferred DC-Chol analogue for *in vivo* studies and application is *N*¹⁵-cholesteryloxycarbonyl-3,7,12-triaza-1,15-pentadecane-diamine (CTAP) 17a'. This compound is a novel pentamine of a type not previously shown to transfect cells. The efficacy of this compound appears to meet

30

the necessary levels apparently required for a cytofectin to have realistic potential clinical use.^[21,24,25,32]

SUMMATION

5

10

15

In summation, in the above examples liposomes were prepared from compounds comprising a cholesterol component and a head group component. The design of the compounds, which were used as cationic lipids, concentrated on the direct manipulation of the head group. With these compounds, the presence of the carbamoyl linkage is believed to be advantageous for low cytotoxicity and the presence of cholesterol is believed to be advantageous for the stabilisation of the liposomal bilayer. In these studies, we were able to alter the identity of the head group in order to increase the net positive charge of the liposome. Increasing the net positive charge is advantageous because it is believed to increase the DNA binding ability and the efficiency of gene transfer of the resultant liposome.

Other modifications of the present invention will be apparent to those skilled in the art.

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CLAIMS

1. A compound capable of acting as a cationic lipid, the compound comprising a cholesterol group having linked thereto a head group; and wherein the head group is more positive than the head group of DC-Chol; but wherein the compound is not synthesised by reacting spermidine and cholesterol chloroformate in CH_2Cl_2 in the presence of *N,N*-diisopropylethylamine.
5
2. A compound according to claim 1 wherein the cholesterol group is cholesterol.
10
3. A compound according to claim 1 or claim 2 wherein the cholesterol group is linked to the head group *via* a carbamoyl linkage.
4. A compound according to any one of claims 1 to 3 wherein the head group is a polyamine group.
15
5. A compound according to claim 4 wherein the polyamine group is a naturally occurring polyamine.
6. A compound according to claim 5 wherein the polyamine is any one of spermidine, spermine or caldopentamine.
20
7. A compound according to any one of claims 1 to 6 in admixture with or associated with a nucleotide sequence.
25
8. A process of preparing a compound according to any one of claims 1 to 7 comprising reacting a cholesterol group with a head group.
9. A process according to claim 8 wherein the process comprises at least one step utilising aza-Wittig methodology.
30

10. A compound according to any one of claims 1 to 7 or a compound when prepared by the process of claim 8 or claim 9 for use in therapy.

5 11. Use of a compound according to any one of claims 1 to 7 or a compound when prepared by the process of claim 8 or claim 9 in the manufacture of a medicament for the treatment of genetic disorder or condition or disease.

10 12. A cationic liposome formed from the compound according to any one of claims 1 to 7 or a compound when prepared by the process of claim 8 or claim 9.

13. A method of preparing a cationic liposome comprising forming the cationic liposome from the compound according to any one of claims 1 to 7 or a compound when prepared by the process of claim 8 or claim 9.

15 14. A cationic liposome according to claim 12 or a cationic liposome as prepared by the method of claim 13 for use in therapy.

20 15. Use of a cationic liposome according to claim 12 or a cationic liposome as prepared by the method of claim 13 in the manufacture of a medicament for the treatment of genetic disorder or condition or disease.

25 16. A combination of a nucleotide sequence and any one or more of: a compound according to any one of claims 1 to 7, a compound when prepared by the process of claim 8 or claim 9, a cationic liposome according to claim 12, or a cationic liposome as prepared by the method of claim 13.

17. A combination according to claim 16 for use in therapy.

30 18. Use of a combination according to claim 17 in the manufacture of a medicament for the treatment of genetic disorder or condition or disease.

19. A pharmaceutical composition comprising a compound according to any one of claims 1 to 7 or a compound when prepared by the process of claim 8 or claim 9 admixed with a pharmaceutical and, optionally, admixed with a pharmaceutically acceptable diluent, carrier or excipient.

5

20. A pharmaceutical composition comprising a cationic liposome according to claim 12 or a cationic liposome as prepared by the method of claim 13 admixed with a pharmaceutical and, optionally, admixed with a pharmaceutically acceptable diluent, carrier or excipient.

10

21. A compound or a cationic liposome substantially as described herein and with reference to any one of the Figures.

22. A process substantially as described herein and with reference to any one of the Figures.

15

1 / 23

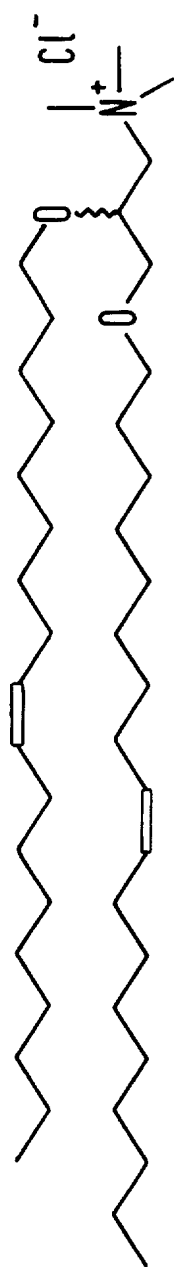
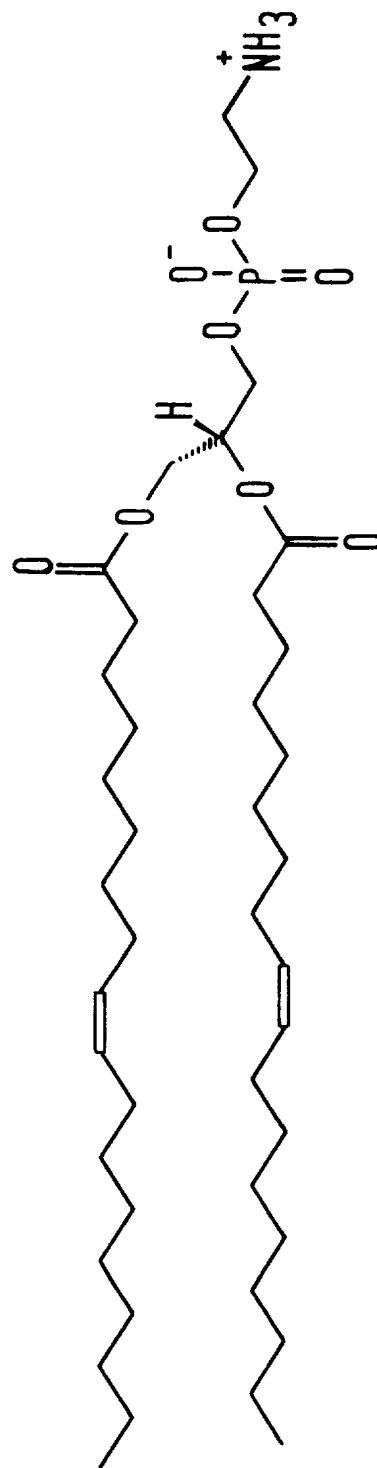
DOTMA *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethyl ammonium chloride

FIG. 1

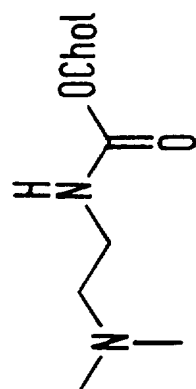


DOPE Dioleoylphosphatidyl ethanolamine

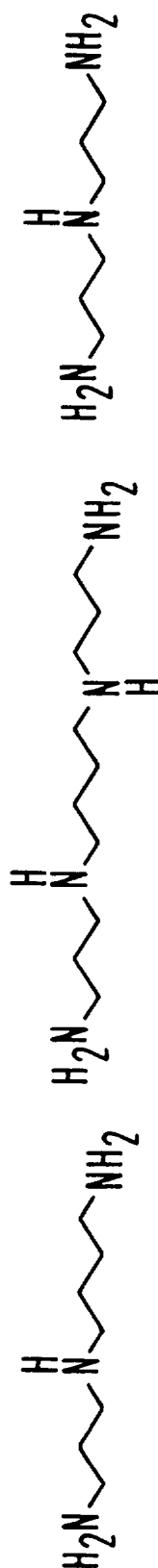
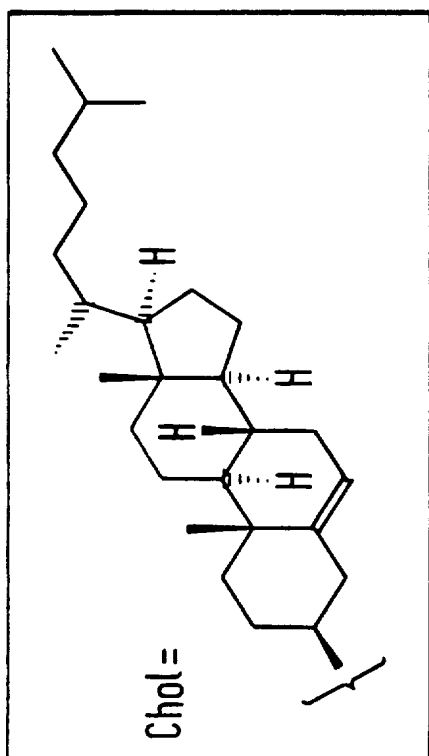
FIG. 2

2 / 23

FIG. 3



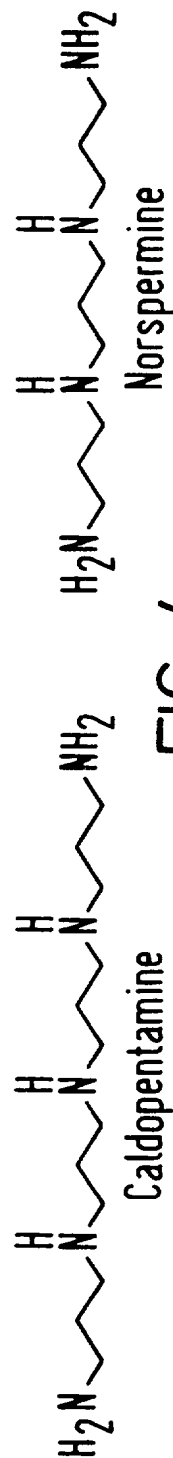
3β-[(N,N-dimethylaminoethyl)carbamoyl]cholesterol
DC-Chol



Spermidine

Spermine

Norspermidine



Caldopentamine

Norspermine

FIG. 4

3 / 23

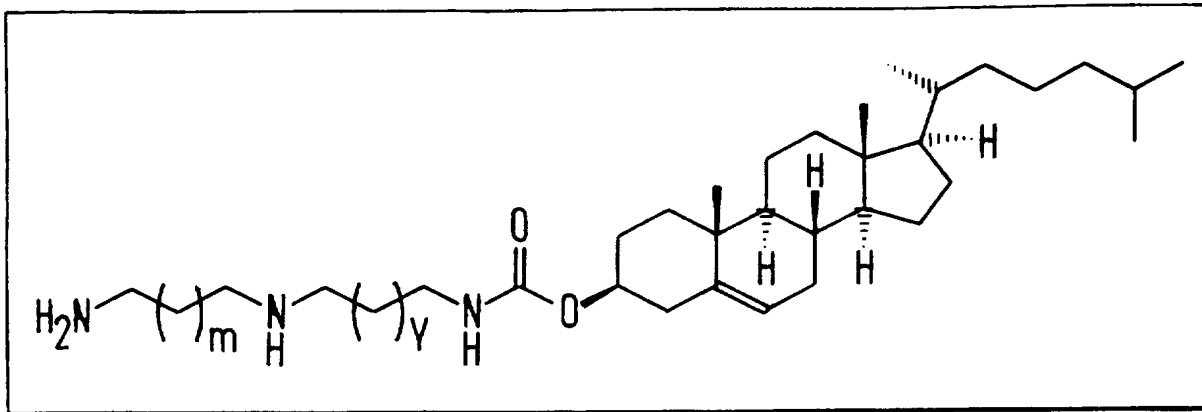


FIG. 5

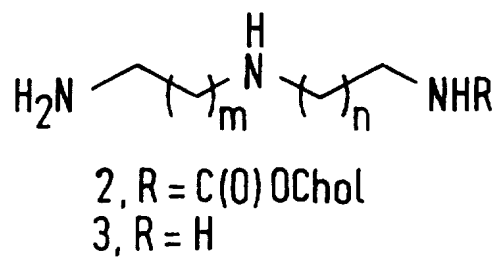
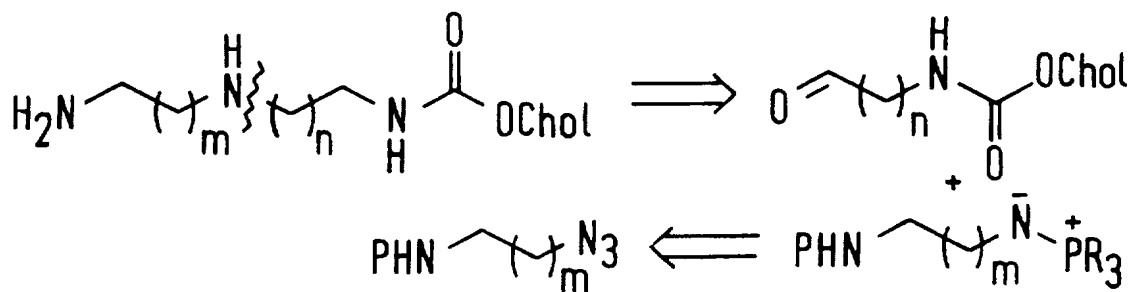
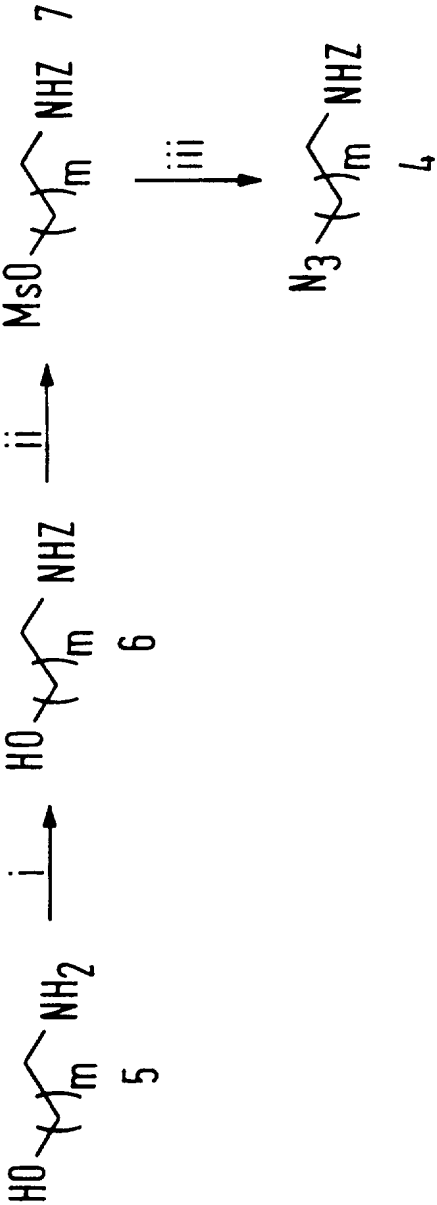


FIG. 6



Scheme 1

FIG. 7

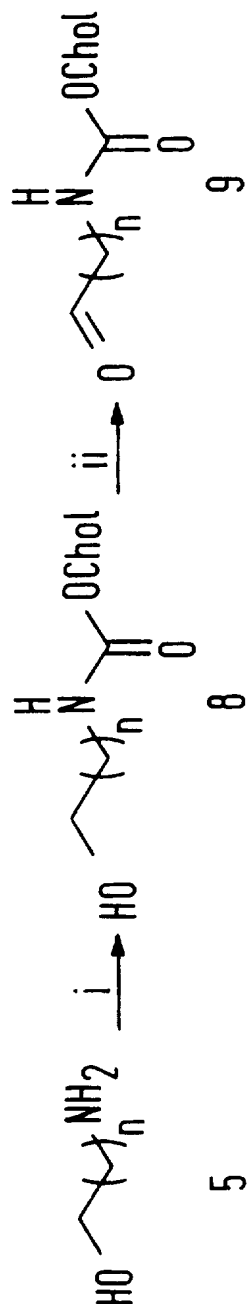


Scheme 2 Reagents and conditions: i, CH_2Cl_2 (0.2M), $\text{PhCH}_2\text{OC(O)Cl}$ (0.45 eqv), 10h; ii, CH_2Cl_2 (0.2M), Et_3N (3 eqv), $\text{CH}_3\text{SO}_2\text{Cl}$ (2.5 eqv) 0°C to r.t., 15min; iii, DMF (0.15M), NaN_3 (5 eqv), NaI, 80°C, 2h

	6a	6b	6c	7a	7b	7c	4a	4b	4c
m	1	2	3	1	2	3	1	2	3
YIELD / %	89	86	92	97	88	95	98	90	96

FIG. 8

Table 1

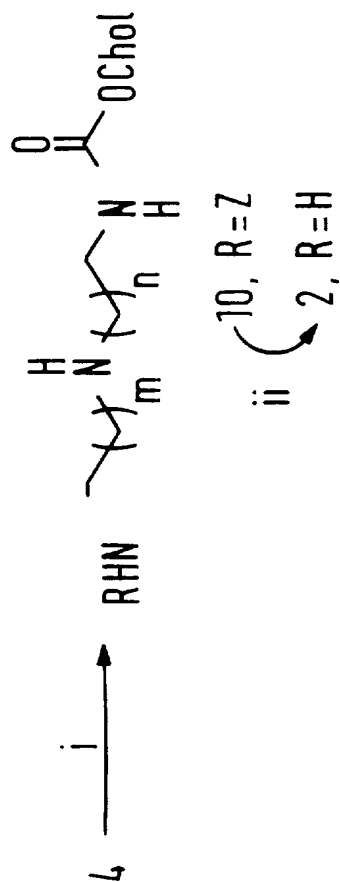


Scheme 3 Reagents and conditions: i, CH_2Cl_2 (0.2M), CholOC(O)Cl (0.45 eqv), 5h; ii, a, CH_2Cl_2 (0.1M) (COCl_2)₂ (1.5 eqv), DMSO (3 eqv), -78°C , 15min; b, **8**, 15 min; c, *i*-Pr₂NEt (3 eqv) to r.t.

	8a	8b	9a	9b
n	1	2	1	2
YIELD / %	98	99	97	93

Table 2

9
G.
F

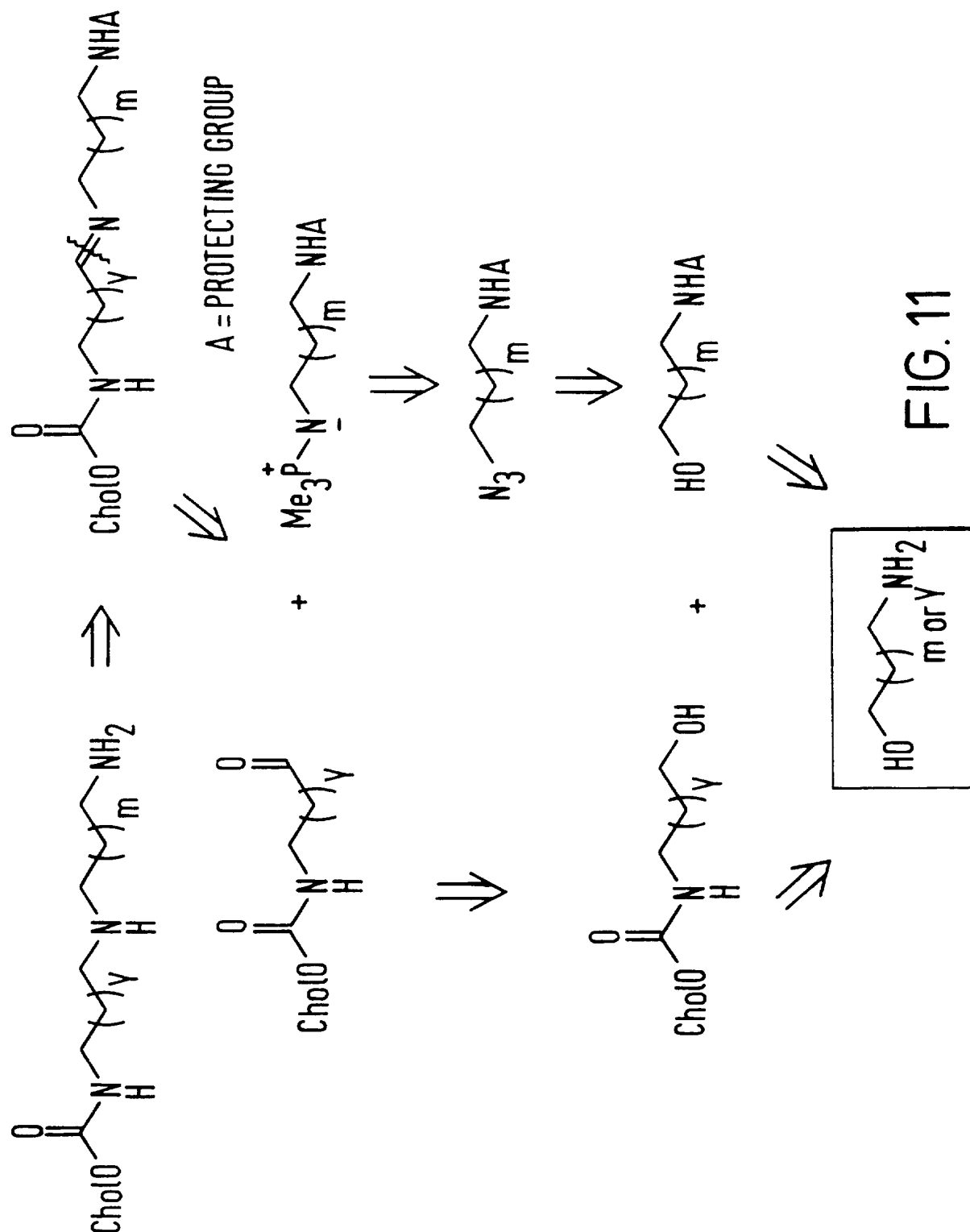


Scheme 4 Reagents and conditions: i, a, THF (0.5M), 4 Å molecular sieves, PMe₃ (1.15 eqv), 30 min; b, **9** (1.1 eqv), 3h; c, EtOH (0.5M), NaBH₄ (2 eqv), 20 h; ii, EtOH (0.2M), c-C₆H₁₀ (20 eqv), 10% Pd(C) (0.5 eqv), reflux, 30 min

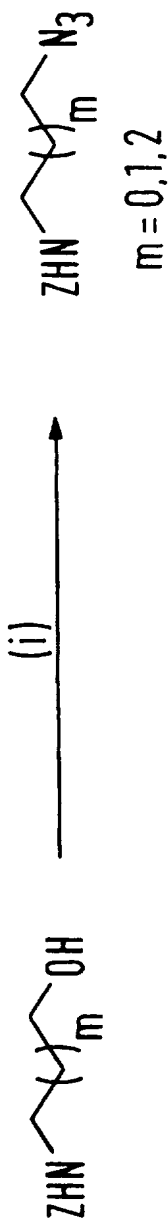
	10a	10b	10c	10d	10e	10f	2a	2b	2c	2d	2e	2f
m	1	2	3	1	2	3	1	2	3	1	2	3
n	1	1	1	2	2	2	1	1	1	2	2	2
YIELD / %	79	72	89	83	87	90	99	99	99	99	99	99

Table 3

FIG. 10



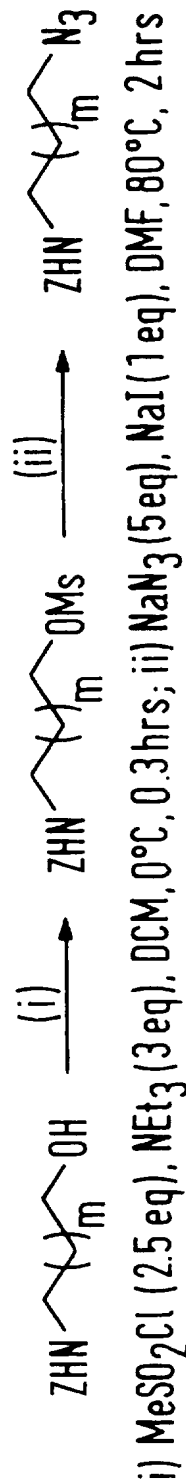
8 / 23

AZIDE FORMATION

i) PPh_3 (2 eq), DIBAL (2 eq), $\text{ZnN}_6 \cdot 2 \text{ Py}$ (0.75 eq), toluene, 2 hrs

m	yield (%)
0	87
1	92
2	90

FIG. 12

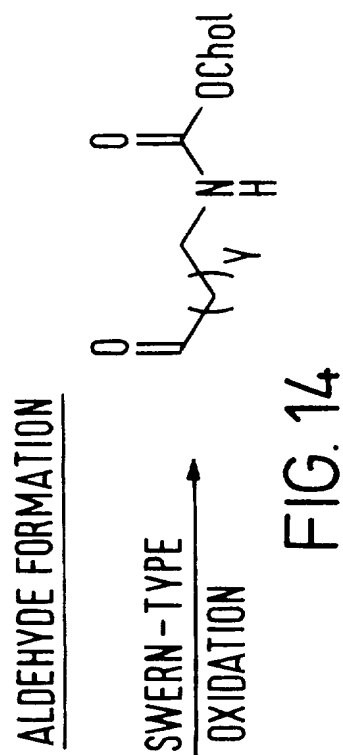


m	Overall yield (%)
0	95
1	79
2	91

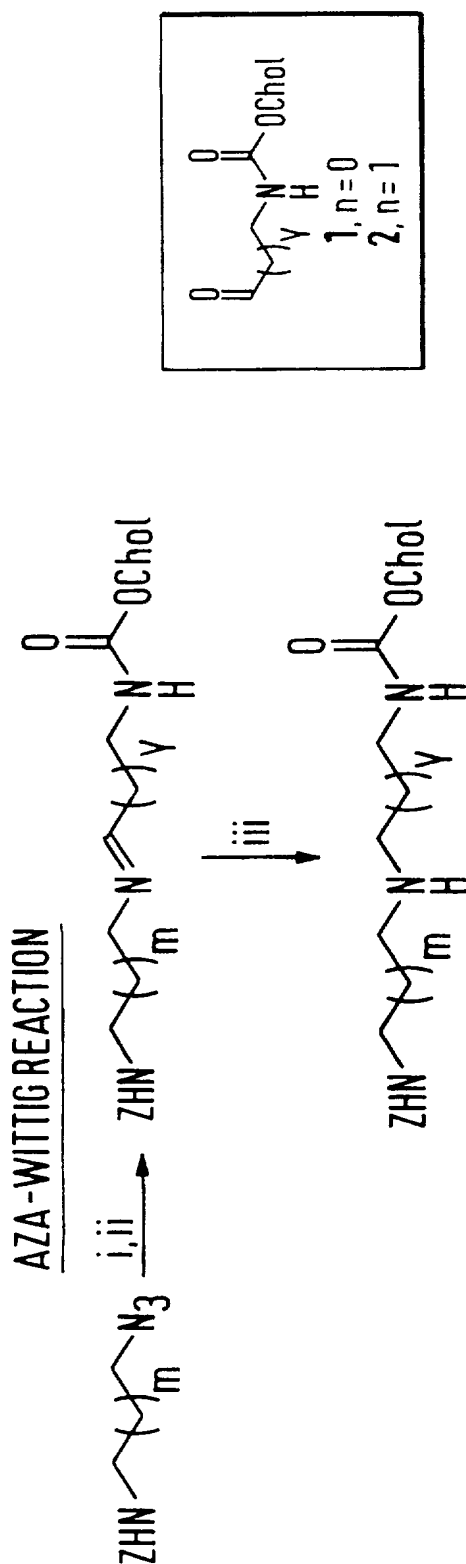
FIG. 13

9 / 23

Y	yield (%)
0	97
1	93



10 / 23



i) PMe_3 (1.1 eq), 4\AA MS (1 g/mmol), THF, 0.75 hrs; ii) **1** or **2** (1.2 eq) in THF, 5-18 hrs;
 iii) NaBH_4 / diglyme (2.0 eq), EtOH, 24 hrs

m	V	Yield (%)
0	0	79
1	0	72
2	0	89
0	1	83
1	1	87
2	1	90



FIG. 15

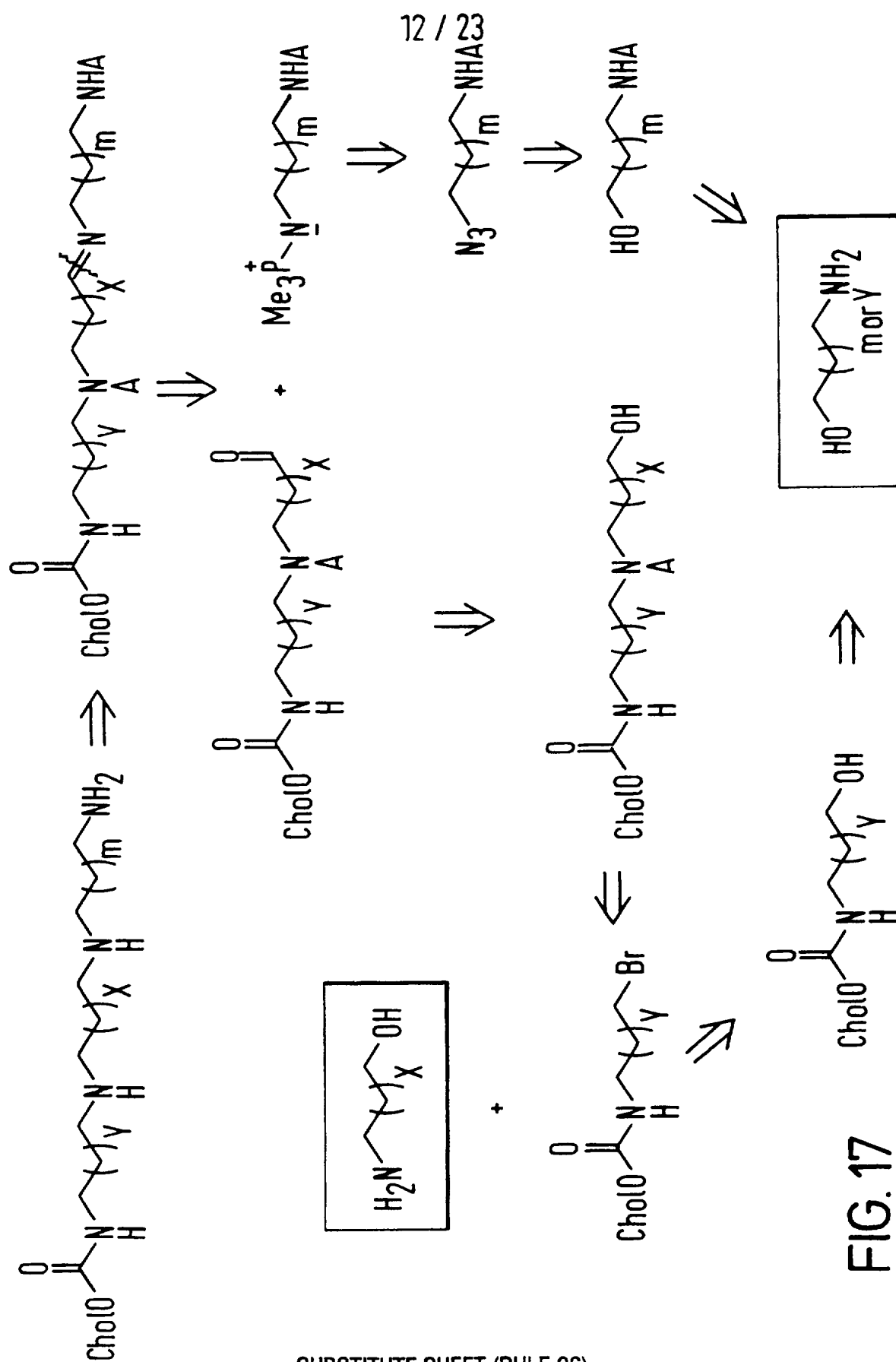


FIG. 17

14 / 23

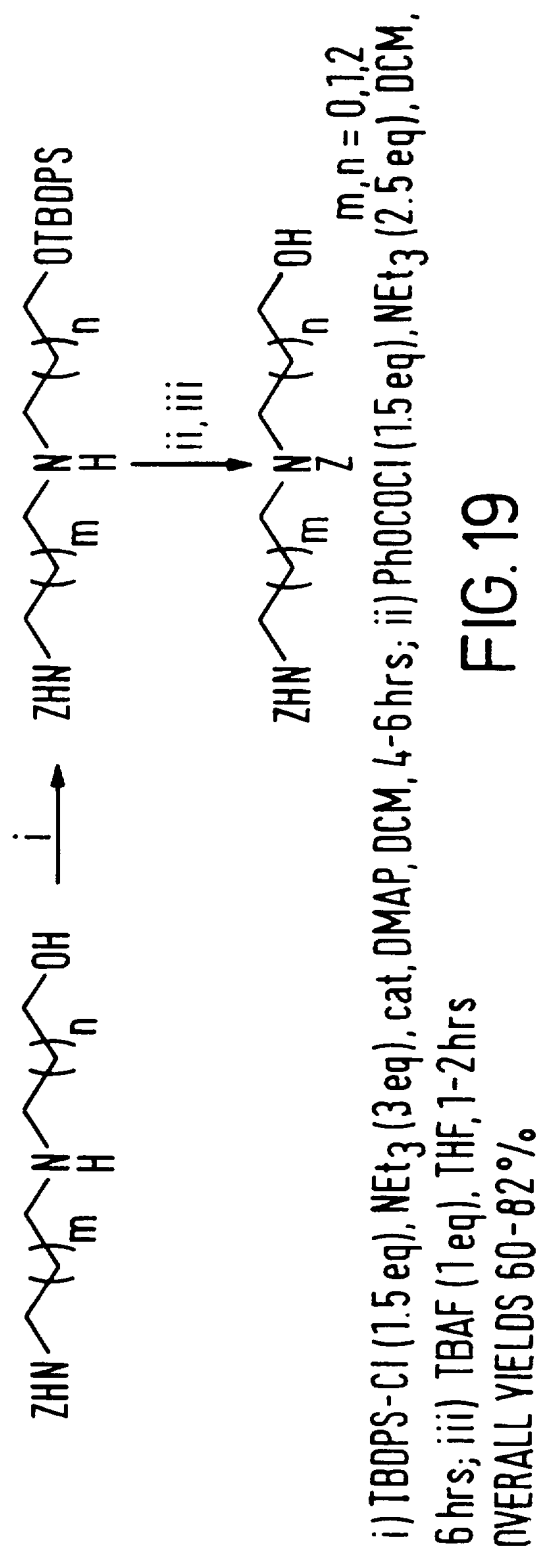
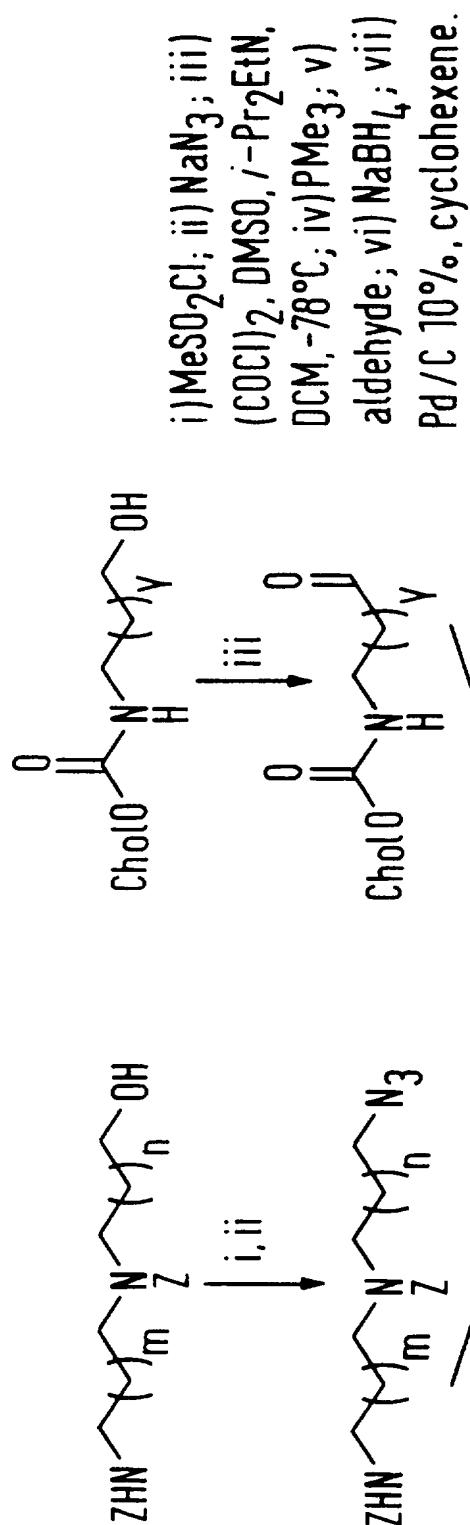


FIG. 19

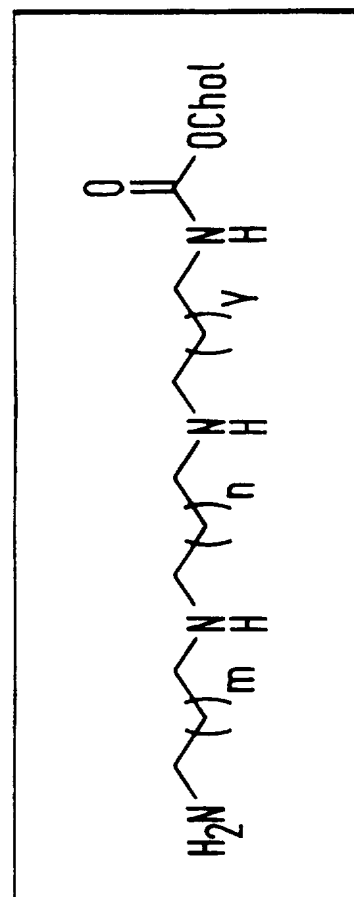
15 / 23

APPLICATION TO SYNTHESIS OF SPERMINOLIPIDS



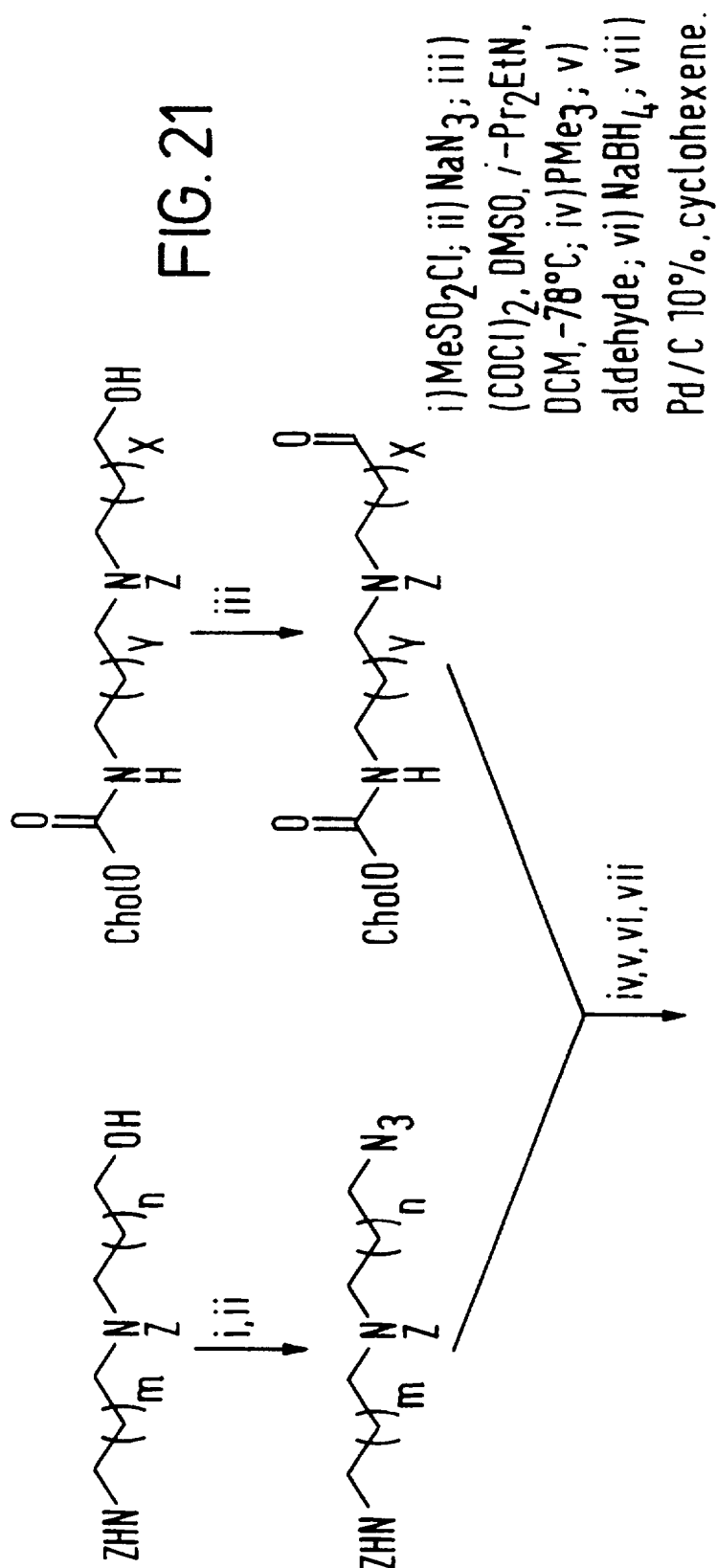
m	n	y	Yield (%)
0	1	0	54
0	1	1	69
0	2	1	80
1	2	1	71
1	0	0	64

FIG. 20

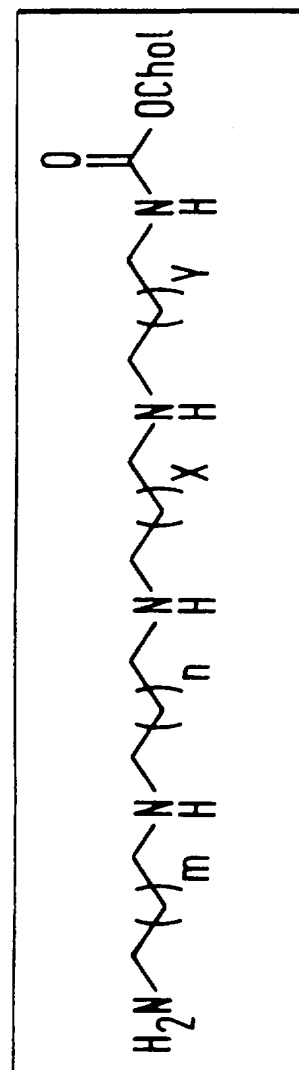


16 / 23

APPLICATION TO SYNTHESIS OF PENTAMINOLIPIDS



m	n	X	Y	Yield (%)
1	2	2	1	56
0	1	2	1	74



17 / 23

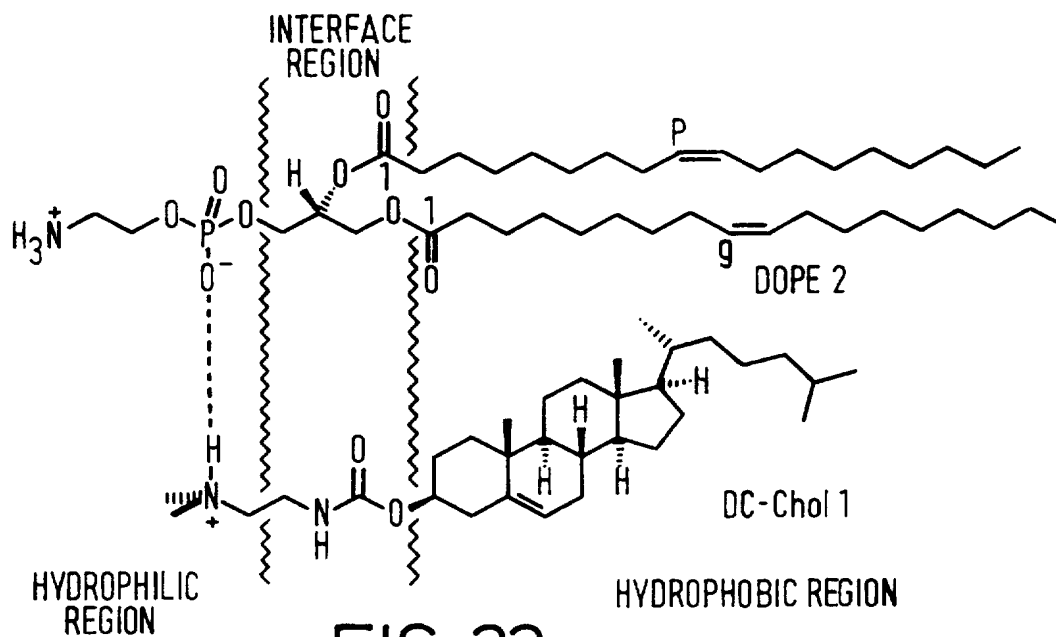


FIG. 22

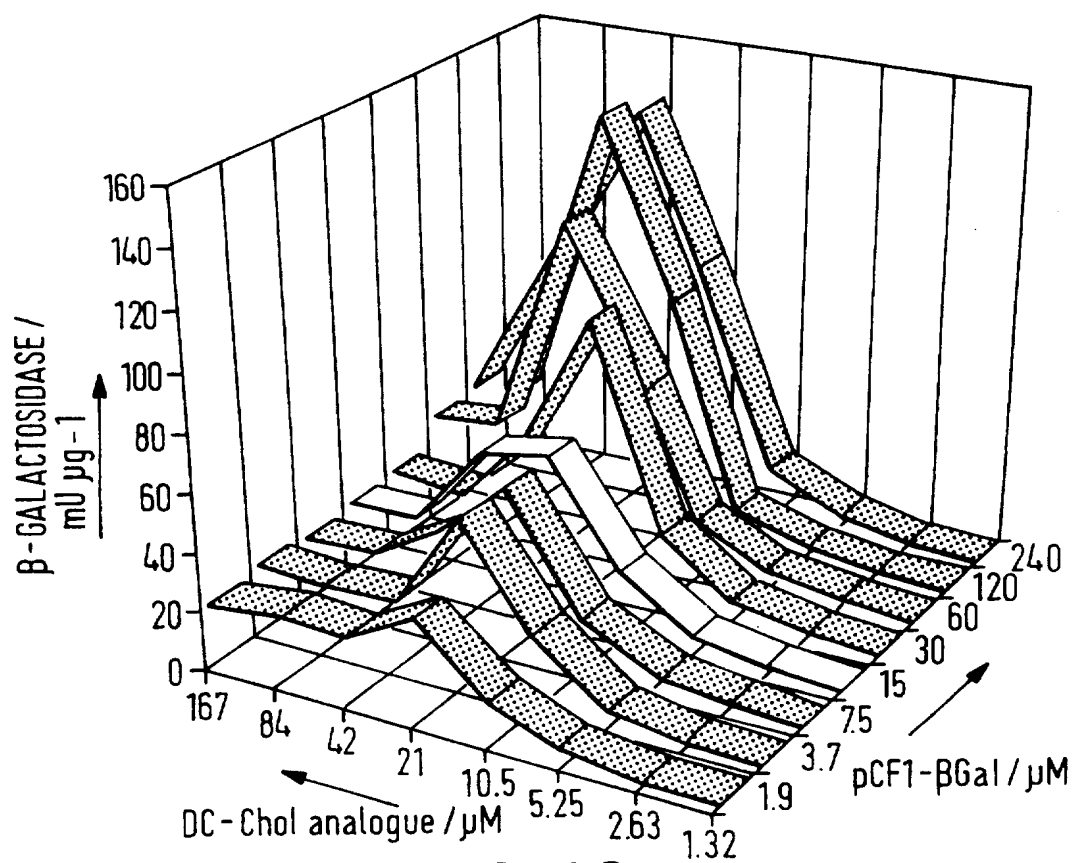


FIG. 23

SUBSTITUTE SHEET (RULE 26)

18 / 23

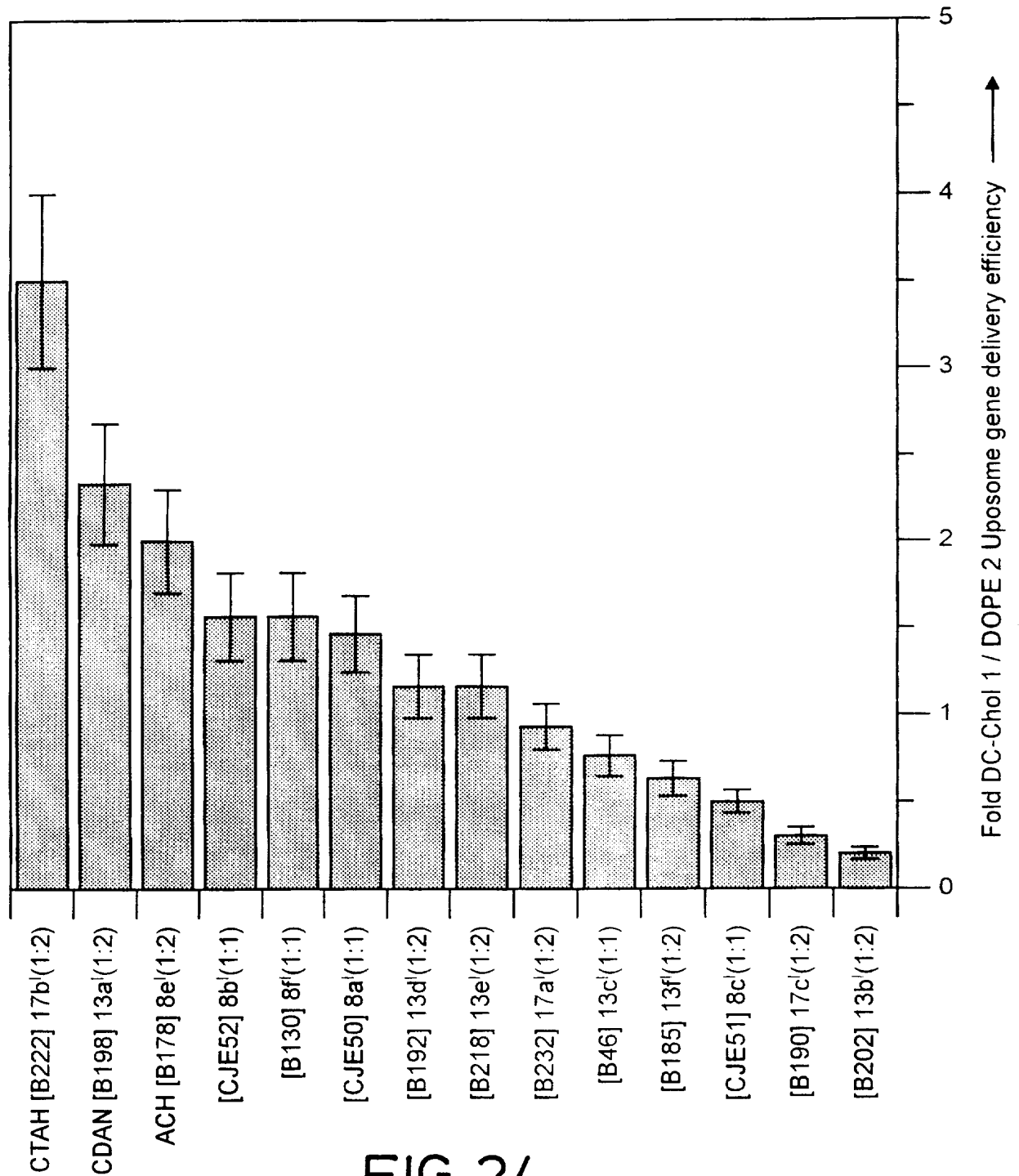


FIG. 24

19 / 23

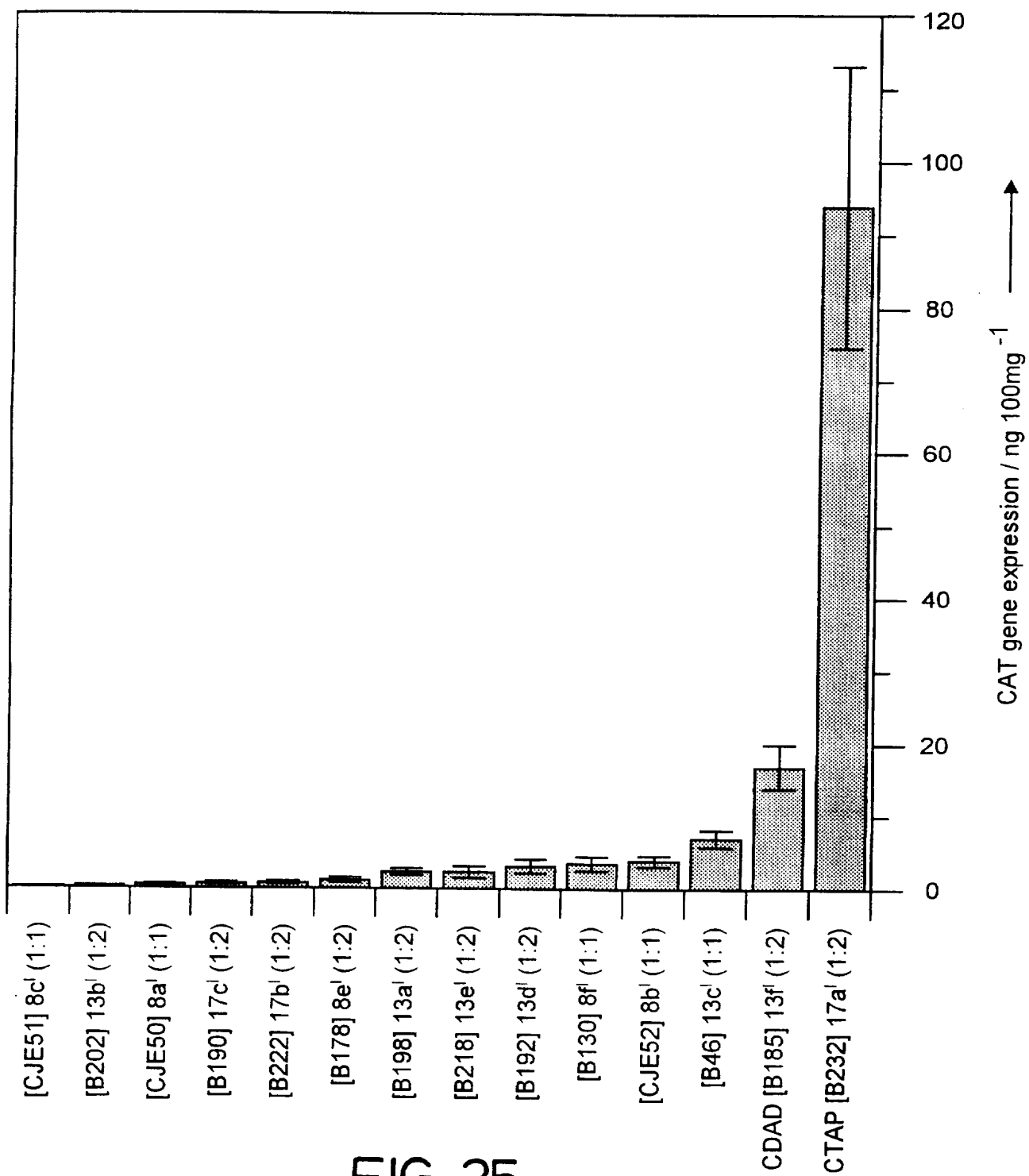
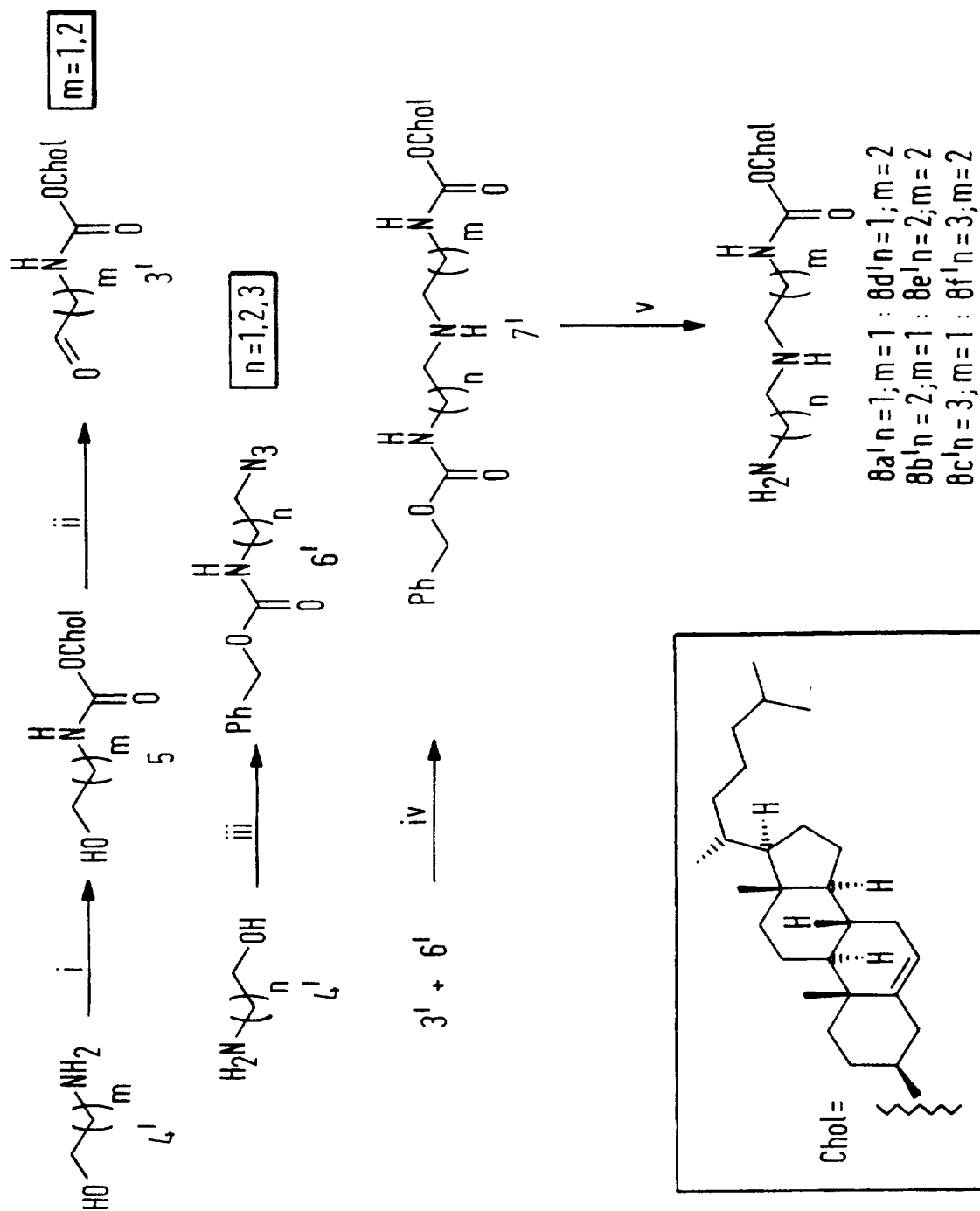


FIG. 25



SCHEME A

FIG. 26

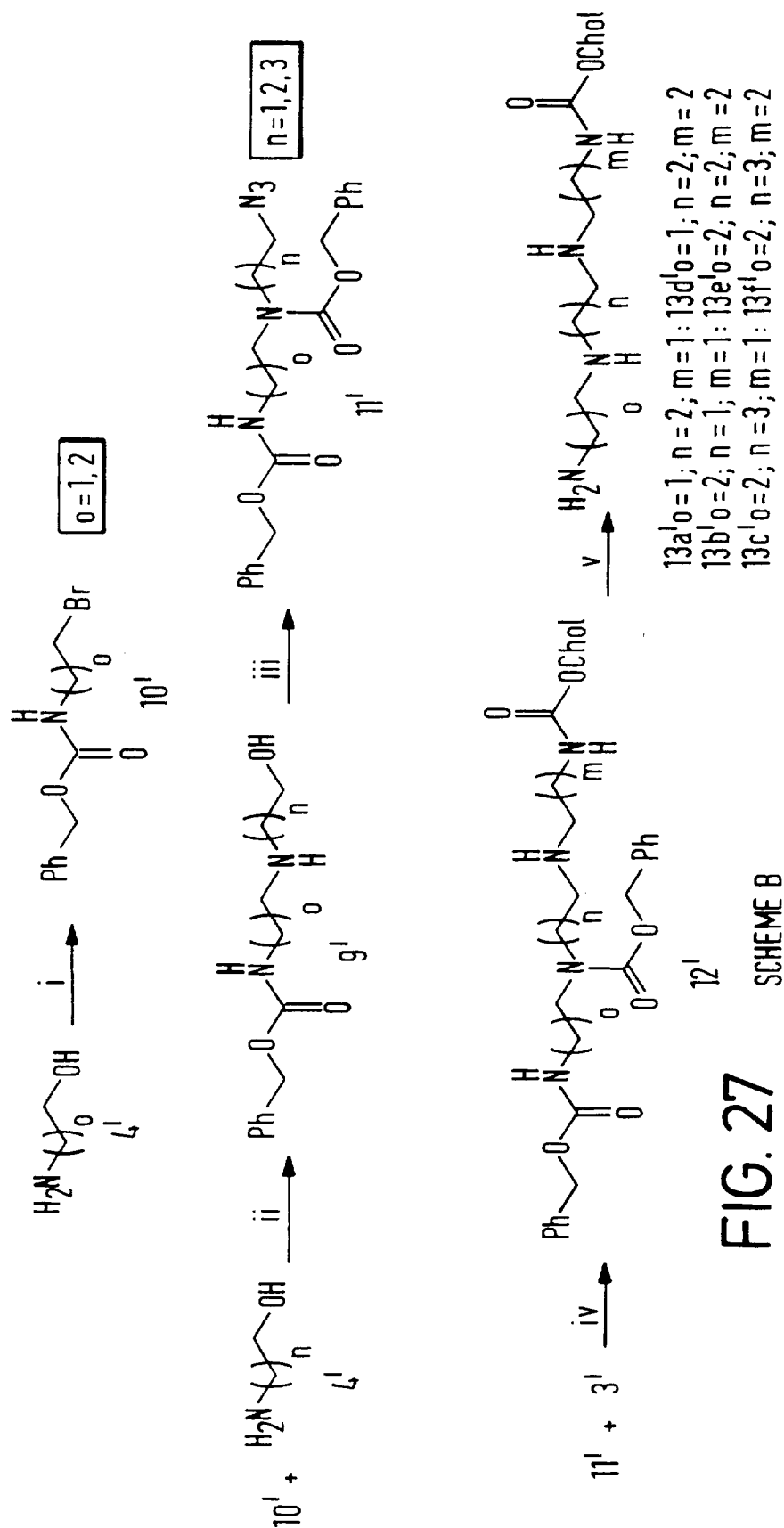


FIG. 27

SCHEME B

22 / 23

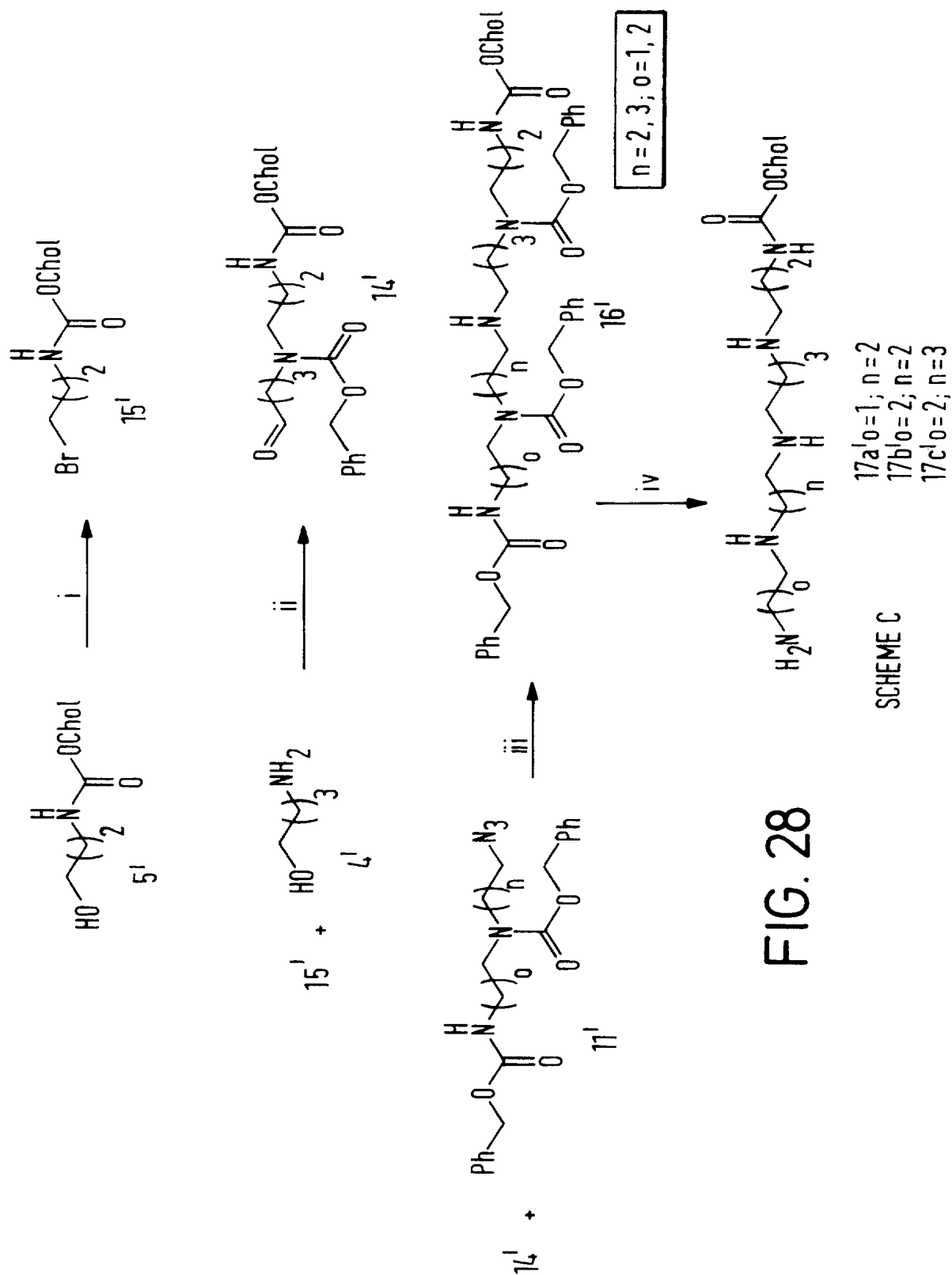


FIG. 28

SCHEME C

23 / 23

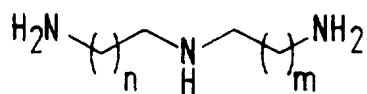
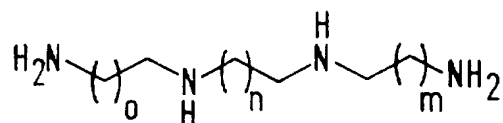
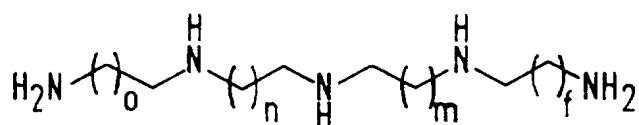
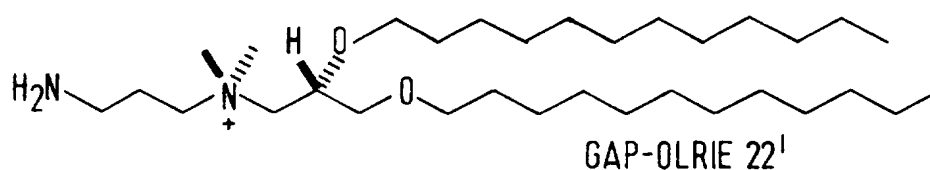
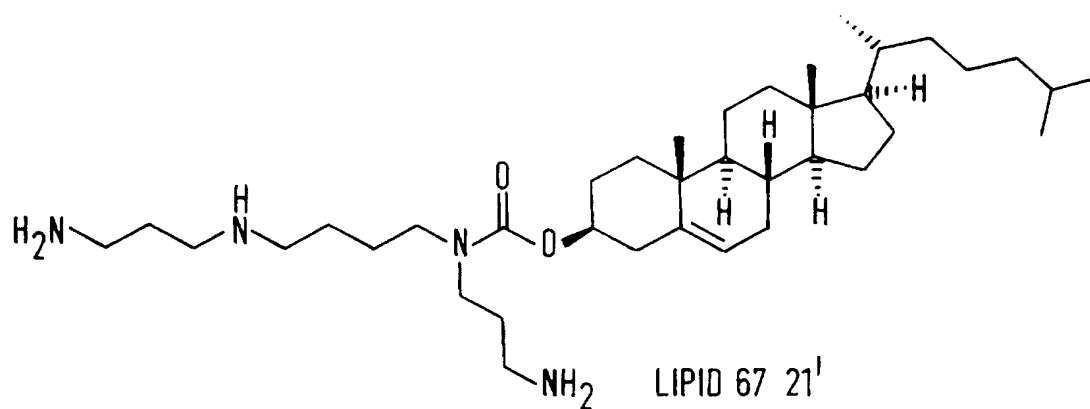
18^I n = 3; m = 219^I o = 2; n = 3; m = 220^I f, m, n, o = 2

FIG. 29

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 97/01426

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07J41/00 A61K9/127 A61K31/575 C12N15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07J A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 10038 A (APOLLON INC ;BOUTIN RAYMOND H (US)) 4 April 1996	1-8, 10-20
Y	see examples 6,10-12,25 ---	1-20
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 221, no. 1, 5 April 1996, pages 82-88, XP000608431	1-7,10, 11,16-19
Y	MORADPOUR D ET AL: "EFFICIENT GENE TRANSFER INTO MAMMALIAN CELLS WITH CHOLESTERYL-SPERMIDINE" see the whole document ---	1-20
	-/--	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search

22 July 1997

Date of mailing of the international search report

- 4 -08- 1997

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Watchorn, P

INTERNATIONAL SEARCH REPORT

Int. Patent Application No.

PCT/GB 97/01426

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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P,X	--- WO 96 40726 A (GENTA INC) 19 December 1996 see page 8 - page 12; figure 5; example 1 see page 16, paragraph 2	1-8, 10-20
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Int. Patent Application No.
PCT/GB 97/01426

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 97/01426

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Please see attached sheet ./.
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/

Claims not searched 21 and 22

Claims 21 and 22 depend for their subject matter on the description and figures in contravention of Rule 6.2(a) - PCT. In addition the qualification "substantially" makes it unclear which subject matter of description and figures is to be included therein. Claims 21 and 22 are therefore so unclear under Art. 6 - PCT - as to be unsearchable - Art. 17(2)(a)(ii) - PCT.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 97/01426

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